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ESTUDO DA ATIVIDADE CITOTÓXICA DA PROTEÍNA DERMONECRÓTICA DO VENENO DE ARANHA MARROM (*Loxosceles intermedia*) COM ÊNFASE NO EFEITO NEFROTÓXICO

Dissertação apresentada como requisito à obtenção do grau de Mestre em Biologia Celular e Molecular, Programa de Pós-Graduação do Departamento de Biologia Celular, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

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Aos meus pais e irmãos, pelo estímulo e amor incondicional,
pela compreensão e liberdade em todas as minhas decisões.
Dedico a realização deste projeto a vocês!

"Tenho várias caras. Uma é quase bonita, outra é quase feia.
Sou um o quê? Um quase tudo."
Clarice Lispector

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"O que eu espero Senhores, é que depois de um razoável período de discussão, todos concordem comigo."

Winston Churchill

SUMÁRIO

RESUMO

Os acidentes envolvendo aranhas marrons (*Loxosceles*) tornaram-se um problema de saúde pública no Brasil e, por conseguinte, de grande relevância médica. O conjunto de sinais e sintomas observados em decorrência da picada de aranha marrom é denominado Loxoscelismo; o qual se caracteriza pelo desenvolvimento de lesão dermonecrótica e espalhamento gravitacional no local da picada (quadro local ou cutâneo) e, menos freqüentemente, distúrbios hematológicos e disfunção renal (quadro sistêmico ou cutâneo-visceral), maiores responsáveis pelos óbitos das vítimas. O conteúdo do veneno de *Loxosceles* e os mecanismos pelos quais induz tais alterações não está bem esclarecido e permanece sob investigação em seus vários segmentos. A presente dissertação tem intuito de aprofundar os conhecimentos quanto à ação citotóxica do veneno loxoscélico, destacando-se o efeito sobre a função renal. Alguns autores atribuem a falência renal como conseqüência das alterações da hemostase (principalmente, a anemia hemolítica) as quais têm sido descritas pelos dados clínicos como proteinúria, hematúria e hemoglobinúria. Além disso, a intensa reação inflamatória observada no local da picada, poderia também induzir a deposição de imunocomplexos que alterariam o fluxo de filtração glomerular e pelos túbulos renais, o que acarretaria na insuficiência renal aguda. Contudo, a literatura científica tem relatado que o veneno exerce ação direta sobre eritrócitos e plaquetas, induzindo hemólise intravascular, agregação plaquetária e coagulação intravascular disseminada; possui efeito inibitório sobre neutrófilos *in vitro*; além de efeitos citotóxicos em células endoteliais de cordão umbilical e de vasos sangüíneos de coelho. Tendo em vista todas essas informações, nós levantamos a hipótese de uma ação direta do veneno sobre as estruturas renais, visto que não existem evidências experimentais de que o veneno interaja com o tecido renal. Para tanto, utilizamos camundongos expostos ao veneno loxoscélico (modelo animal que não desenvolve a lesão dermonecrótica) e foi observada a indução de lesão renal. Biópsias renais destes animais em microscopia de luz evidenciaram alterações morfológicas contundentes que incluem acúmulo de material eosinofílico e eritrócitos extravasculares na cápsula de Bowman, colapso glomerular, citotoxicidade das células e deposição de material proteináceo no lúmen dos túbulos. Em microscopia eletrônica de transmissão, as alterações morfológicas incluem citotoxicidade de células epiteliais e endoteliais glomerulares e distúrbios na membrana basal renal, bem como alterações dos túbulos que apresentaram vacúolos na membrana citoplasmática, distúrbios mitocondriais, aumento do retículo liso, presença de autofagossomos. Nós observamos também que o veneno causa azotemia, com elevação do nível de uréia sangüíneo, embora não fora observada alteração dos níveis séricos de complemento C3 ou hemólise. Por meio de reações de imunofluorescência por microscopia confocal empregando anticorpos que reconhecem as proteínas do veneno, detectamos a ligação direta de toxinas loxoscélicas em estruturas renais. Ao se realizar dupla marcação de veneno, núcleo (por citoquímica com DAPI) e colágeno tipo IV e laminina, demostramos a deposição das toxinas em células epiteliais glomerulares e tubulares e em membrana basal, mas não houve marcação dos núcleos das células renais. Os dados da ligação direta da toxina ao rim foram reforçados pela constatação de que o veneno é rico em proteínas básicas e de baixa massa molecular em eletroforese bidimensional, o que explicaria o acesso (baixa massa) e a presença de toxinas loxoscélicas na membrana basal (aniônica).

Em adição, houve a detecção de toxinas por "imunoblotting" na região de 30kDa de extrato de membrana de rins envenenados. Tais resultados corroboraram para a prospecção de uma possível ação citotóxica sobre linhagens celulares tumorais, as quais foram avaliadas quanto a morfologia celular, viabilidade e proliferação celular (pelos métodos de azul de Trypan e MTT, respectivamente), em concentrações e tempos crescentes. Todas as células assumiram uma forma arredondada e, em alguns casos, houve perda da adesividade ao substrato de cultura. No entanto, de modo geral, não foi observada alteração da viabilidade celular e a taxa de proliferação celular foi bastante variada entre as linhagens expostas ao veneno de *Loxosceles*. Por meio de técnicas de imunofluorescência por microscopia confocal e microscopia de luz com marcação de PAS, selecionamos uma das linhagens (MCF-7) com intuito de avaliar as alterações morfológicas desencadeadas pelo veneno. Os pontos de adesão focal e a organização dos filamentos de actina foram perturbados, bem como, observou-se a ligação das toxinas do veneno na superfície celular. Além disso, o tratamento com o veneno também degradou a matriz extracelular. Os resultados apontaram citotoxicidade do veneno de aranha marrom sobre a morfologia das células tumorais e, por conseguinte, possibilitam a utilização das toxinas do veneno como ferramentas viáveis para a pesquisa em Biologia Celular. A ação direta das toxinas do veneno de aranha marrom foi então novamente avaliada, desta vez utilizando uma proteína recombinante do veneno (*LiRecDT*), com ênfase no seu possível efeito nefrotóxico, como descrevemos para o veneno bruto. Nós postulamos que a toxina dermonecrótica (esfingomielinase-D), que é a proteína bioquímica e biologicamente melhor caracterizada do veneno de aranha marrom, poderia estar envolvida diretamente ao dano renal, pois suas características de carga e massa molecular são similares aos dados que obtivemos na detecção de proteínas do veneno bruto ligadas ao rim. Nesse ínterim, utilizando camundongos expostos a *LiRecDT*, nós evidenciamos a indução direta de injúria renal. Os achados histopatológicos de biópsias renais demonstraram alterações contundentes que incluem edema glomerular e necrose tubular. Houve deposição de material proteináceo no lúmen dos túbulos, vacuolização e lise de células epiteliais. Não foi visualizada infiltração de leucócitos em glomérulos e túbulos renais, bem como os vasos renais não evidenciaram sinais de resposta inflamatória. Na imunofluorescência por microscopia confocal foi demonstrada a ligação direta de *LiRecDT* no tecido renal. Por ensaio de "imunoblotting" de extrato de membrana renal de camundongo tratado com *LiRecDT*, que revelou a presença de um sinal positivo na região de 33-35 kDa, fato que fortalece a idéia de ação nefrotóxica direta da toxina dermonecrótica. A utilização de cultura de células epiteliais de rim canino (MDCK) permitiu novamente a observação da ligação de *LiRecDT* quando observadas por imunofluorescência em microscopia confocal. Similarmente, o tratamento de células MDCK com *LiRecDT* em cultura determinou alterações morfológicas de vacuolização citoplasmática, mudança do comportamento do perfil de espalhamento celular e adesão célula-célula. Em adição, a viabilidade celular foi avaliada pelo método de captação por endocitose de corante vital (Vermelho Neutro) e ensaio de XTT, sendo a redução dependente da concentração e tempo de exposição. Os presentes resultados corroboram para a idéia de que a toxina dermonecrótica, assim como o veneno bruto, denota em nefrotoxicidade direta sobre as células renais *in vivo* e *in vitro*. Os trabalhos decorrentes deste projeto contribuem para o entendimento dos mecanismos pelos quais os venenos de aranhas marrons desencadeiam um efeito citotóxico e, em especial, nefrotóxico observado no Loxoscelismo.

1. INTRODUÇÃO

1.1 Aranhas

As aranhas são animais classificados taxonomicamente como pertencentes ao grupo dos artrópodes terrestres, classe dos aracnídeos, a qual é a segunda no número de espécies, sendo que as aranhas compõem seu maior subgrupo. Representam um grupo bastante antigo, o fóssil mais velho que se tem conhecimento data de 300 milhões de anos atrás. Apresentam cerca de 40.000 espécies descritas (PLATNICK, 1993). Do ponto de vista morfológico, possuem o corpo dividido em dois segmentos, o cefalotórax (união entre cabeça e tórax) e o abdômen. Exibem quatro pares de patas, quelíceras, pedipalpos e podem possuir até oito olhos (RUPPERT E BARNES, 1996).

Há dois grupos principais de aranhas: o Orthognatha ou migalomorfa (aranha primitiva ou aranha “trapdoor”) cuja quelícera se projeta à frente partindo do cefalotórax enquanto as presas posicionam-se para baixo. Os representantes do grupo Labdognatha ou araneomorfa possuem as quelíceras posicionadas verticalmente e que conjuntamente com as presas se movem lateralmente como pinças (RASH E HODGSON, 2002). Neste grupo está incluído o gênero Loxosceles, a “aranha marrom”.

As espécies mais perigosas de aranhas são encontradas no grupo das Labidognathas (araneomorfas): *Lactrodectus* (viúva negra – Theridiidae), *Loxosceles* (aranha violino ou aranha marrom – Loxoscelidae), *Phoneutria* (aranha de bananeira – *Ctenidae*). Tais aracnídeos são responsáveis por muitos casos graves de envenenamento e registros de óbito. Muitas outras famílias são consideradas perigosas: *Segestriidae*, *Agelenidae*, *Salticidae*, *Gnaphosidae*, *Thomisidae*, *Heteropodidae*, *Clubionidae* e *Lycosidae* (aranha de jardim ou tarântula), embora a maioria das notificações de envenenamentos não seja adequadamente documentada.

Notificações relacionadas com acidentes envolvendo essas espécies devem ser cuidadosamente consideradas a luz do polimorfismo natural da síndrome causada por cada tipo de envenenamento e a incerteza da identificação taxônomica. Como dado, apenas três gêneros (*Atrax, Lactrodectus* e *Loxosceles*) são apontados como responsáveis por acidentes culminando em óbito em humanos (ESCOUBAS *et al.,* 2000).

1.2 Aranhas do gênero *Loxosceles*

As aranhas do gênero *Loxosceles* pertencem à família Loxoscelidae, sub-ordem Labidognatha, ordem Araneida, classe Arachnida e filo Arthropoda (RUPPERT E BARNES, 1996; SOERENSEN, 1996). São popularmente denominadas de aranhas marrons por possuírem um colorido uniforme que varia de marrom claro até o marrom escuro. São aranhas de pequeno porte que possuem comprimento corporal entre 8 e 15mm e suas patas medem de 8 a 30mm. Os machos possuem corpo menor e pernas relativamente mais longas do que das fêmeas. Possuem seis olhos brancos brilhantes reunidos sobre o cefalotórax, em três grupos de dois, em semicírculo. O cefalotórax é baixo, não ultrapassando em altura o abdômen (BÜCHERL, 1972). A organização das glândulas de veneno das aranhas do gênero *Loxosceles* segue a arquitetura geral das glândulas de veneno das aranhas em geral, onde o epitélio secretor é revestido por uma membrana basal e rodeado por uma espessa musculatura (JUNQUA E VACHON, 1968; BÜCHERL, 1972; FOIL *et al.*, 1979; SANTOS *et al.*, 2000).

Figura 1: Aranha marrom (*Loxosceles intermedia*) (aumento de 2x)

Possuem hábitos noturnos e são sedentárias. Preferindo a escuridão, algumas vivem sob pedras, troncos de árvores, restos vegetais, telhas e tijolos

empilhados. Sob condições especiais, adquirem hábitos intradomiciliares, podem ser encontradas atrás de quadros e móveis, no meio de roupas, livros, caixas de papelão e outros objetos. Constróem teias irregulares semelhantes a algodão esfiapado. São carnívoras, alimentando-se de pequenos insetos. Reproduzem-se com facilidade, mesmo em ambientes desfavoráveis, podendo estar presentes em ambientes com temperaturas entre 8 e 43°C. (BÜCHERL E ROSENFELD, 1954; HITE *et al*., 1960; GAJARDO-TOBAR, 1966; LUCAS, 1988). Os acidentes com aranhas marrons ocorrem principalmente durante as estações mais quentes do ano, primavera e verão (SCHENONE E LETONJA, 1975; MANFREDINI *et al*., 1993). Podem sobreviver até 276 dias sem alimento e ambos os sexos são venenosos (LOWRIE, 1980; GERSTCH E ENNIK, 1983; FUTRELL, 1992).

Em geral, as fêmeas são maiores que os machos e o seu veneno possui maior concentração de proteínas. Tal fato associado a grande produção de veneno, intensifica o potencial tóxico do envenenamento pela aranha fêmea. Ao contrário de outros animais peçonhentos, como ofídios, escorpiões e outros quelicerados, a aranha marrom não é agressiva. A maior parte dos acidentes loxoscélicos (nome dado ao acidente provocado pela picada da aranha marrom) é devida à compressão do animal, inadvertidamente, contra a pele no ato de vestir-se, calçar-se, enxugar-se ou durante o sono (SUAREZ *et al.,* 1971; FUTRELL, 1992; DA SILVA *et al*., 2004).

1.3 Epidemiologia do Loxoscelismo

As aranhas do gênero *Loxosceles* são cosmopolitamente distribuídas, adaptadas aos climas tropical, subtropical e temperado, são descritas espécies em todos continentes do mundo (FUTRELL, 1992; DA SILVA *et al*., 2004). No continente americano, aproximadamente 18 espécies ocorrem na América do Norte, América Central e Antilhas e 30 espécies na América do Sul. No Brasil, estão descritas 7 espécies heterogeneamente distribuídas, as quais são: *Loxosceles gaucho* (SP e RS), *Loxosceles similis* (PB, SP e MG), *Loxosceles adelaide* (RJ), *Loxosceles hirsuta* (RS, SC e PR), *Loxosceles amazonica* (AM e CE), *Loxosceles intermedia* (do RS ao RJ) e *Loxosceles laeta* (região Sul e Sudeste).

Os acidentes com aranha marrom tornaram-se, nos últimos 10 anos, um problema de saúde pública de grande interesse para a área médica no Estado

do Paraná, pois os números de ocorrências notificadas vêm aumentando de forma alarmante. Outro fator preocupante é a elevada infestação domiciliar da aranha da espécie *Loxosceles intermedia*. Os acidentes se relacionam aos hábitos adotados pela aranha marrom e tendem a ocorrer principalmente em pessoas do sexo feminino, sendo mais afetadas as regiões dos membros inferiores, superiores e o tronco, caracterizando o acidente como doméstico e ocasionado principalmente como ato de defesa da aranha ao ser comprimida contra o corpo do indivíduo (RIBEIRO *et al*., 1993).

No Brasil, o Estado do Paraná tem sido o que mais notifica acidentes por aranhas marrons. Correspondem a mais de 50% daqueles provocados por aracnídeos notificados no Mistério da Saúde do Brasil. No ano de 2004, até o mês de outubro, foram notificados 3334 casos de acidentes em Curitiba e região metropolitana*. Dados colhidos junto ao SESA/ Centro de Saúde Ambiental do Estado do Paraná, mostram que a espécie predominante é a *L. intermedia*, no entanto, em algumas regiões encontram-se populações menores de *L. laeta* e *L. gaucho*.

A mudança do sistema de informação que acompanha as notificações deste agravo orienta uma análise cautelosa desta elevação. As notificações até então monitoradas por sistema próprio do município passaram a ser informadas no Sistema de Informação de Agravos de Notificação - SINAN, que é o modelo preconizado pelo Ministério da Saúde, alterando assim o processo de vigilância e possibilitando diferenças decorrentes da substituição do sistema. Por outro lado, mudanças climáticas, como a diminuição da duração e intensidade dos períodos de frio na região Sul podem também ter ocasionado o aumento do número de acidentes (SECRETARIA MUNICIPAL DE SAÚDE DE CURITIBA, 2002)

1.4 Loxoscelismo

O quadro clínico desenvolvido no envenenamento induzido por acidentes com aranhas marrons é conhecido como Loxoscelismo (MARTINEZ-VARGAS, 1987; RIBEIRO *et al.*, 1993) e se caracteriza por uma lesão dermonecrótica (REES *et al.,* 1984) no local da picada (quadro cutâneo) e efeitos sistêmicos (quadro cutâneo-visceral ou sistêmico) (BINFORD E CONNOR, 1976; PRATT *et al.*, 1995), entre os quais se destacam os distúrbios

*** comunicação pessoal (CENTRO EPIDEMIOLÓGICO DE CURITIBA, novembro de 2004)**

da hemostase (DENNY *et* al., 1964; REES *et al.*, 1988; ZANETTI *et al.*, 2002; DA SILVA *et al.*, 2003) e da função renal (FUTRELL, 1992; LUNG E MALLORY *et* al., 2000; DA SILVA *et al*., 2004).

O quadro cutâneo representa cerca de 84 a 97% dos casos (BARBARO *et al.*, 1992). A picada, por ser inicialmente pouco dolorosa, geralmente passa despercebida pelo paciente. Após 2 a 8 horas, a dor pode variar de moderada a severa e é descrita como dor local do tipo “queimação” ou ardência. Pode ser acompanhada por prurido, edema, eritema, sensação de mal-estar geral e podendo em alguns casos ocorrer febre. Em seguida, pode surgir uma lesão de 1 a 30cm de diâmetro, circundada por halo vermelho e, em certas ocasiões, por uma zona pálida denominada placa marmórea (RODRIGUES *et al*., 1986; FUTRELL, 1992; DA SILVA *et al.*, 2004). Após 3 a 5 dias do acidente pode ocorrer acúmulo de leucócitos polimorfonucleares, necrose e formação de abscesso (SMITH E MICKS, 1970; OSPEDAL *et al.*, 2002). Em alguns casos a lesão cutânea necrótica evolui em 2 a 6 semanas, com formação de uma escara de difícil cicatrização e pode dar origem a seqüelas deformantes de importância considerável (ZANETTI *et al.*, 2002). O ferimento crônico produzido pela picada da aranha marrom apresenta vasculite mediada por leucócitos (OSPEDAL *et al.*, 2002), que pode produzir lesões como piodermite gangrenosa (REES *et al*., 1988). Esta lesão pode ter como agente sinérgico, acentuando sua gravidade, a presença de microorganismos provenientes das quelíceras que, no momento da picada, são inoculados concomitantemente com o veneno. Um agente importante de infecção secundária à picada é o *Clostridium perfringens*, bacilo gram positivo anaeróbio (MONTEIRO *et al.,* 2002).

O loxoscelismo cutâneo-visceral ou sistêmico ocorre com menor freqüência (3 – 16% dos casos) e é observado apenas nos casos mais graves. As primeiras manifestações aparecem após 24 horas e os sintomas incluem, além da reação local, astenia, febre, episódios eméticos, alterações sensoriais, cefaléia, insônia e nos casos mais graves, convulsões e coma. Pode também ocorrer prurido generalizado e petéquias (MARTINEZ-VARGAS, 1987; SCHENONE *et al*.,1989; FUTRELL, 1992; BRAVO *et al*., 1993).

Nos casos de gravidade ainda maior, as alterações no quadro hematológico incluem anemia hemolítica, agregação plaquetária causando trombocitopenia (BASCUR *et al.,*1982; WILLIAMS *et al.*, 1995) e coagulação

intravascular disseminada (DENNY *et al.,* 1964) as quais podem determinar diminuição do hematócrito, aumento da bilirrubina indireta e icterícia (SCHENONE E SUAREZ, 1978; REES *et al*., 1988; FUTRELL,1992). Outras conseqüências decorrentes do envenenamento incluem alterações vasculares nos pulmões, fígado e rins (PIZZI *et al*., 1957; LUNG E MALLORY, 2000).

Uma das alterações funcionais mais graves, sendo a principal causa de morte nestes acidentes (MINISTÉRIO DA SAÚDE, 1998) é a insuficiência renal aguda (IRA), a qual caracteriza-se por hemoglobinúria e hematúria, e em casos extremos pode levar a obstrução da luz tubular por acúmulo de proteínas ou pelo edema das células epiteliais (IRA oligúrica ou não oligúrica) (WASSERMAN E ANDERSON, 1984; SCHENONE *et al*., 1989; FUTRELL, 1992; SEZERINO, 1998; LUNG E MALLORY, 2000).

A evolução de um dos quadros descritos ou de ambos pode depender de alguns fatores relacionados com a espécie da aranha, sexo do animal (OLIVEIRA *et al.,* 1999), seu estágio de desenvolvimento (ANDRADE *et al.,* 1999), quantidade de veneno inoculada, assim como a idade (SEZERINO *et al.,* 1998) e susceptibilidade individual do acidentado. Outros fatores como o estado nutricional, local da picada, sensibilidade ao veneno e o tempo em que o indivíduo leva para procurar um tratamento adequado são igualmente importantes no desenvolvimento do quadro clínico pós-envenenamento (GAJARDO-TOBAR, 1966; SCHENONE *et al*., 1989; BARBARO *et al*., 1994). A evolução dos sintomas está relacionada à quantidade de veneno inoculada, localização da picada e a condição imunológica do paciente (MORÁN *et al*., 1981; CICARELLI *et al*., 1983/1984; HEREDIA *et al*., 1989). Esta síndrome não tem ligação alguma com sexo e idade do paciente, estação do ano e seriedade da lesão cutânea. Não tem relação entre o tamanho e o tipo da lesão cutânea e o grau do comprometimento visceral. A reação sistêmica não é necessariamente proporcional à reação local e vice-versa, uma vez que os sintomas sistêmicos podem desenvolver-se antes de alguma reação local poder ser notada.

O tratamento do loxoscelismo ainda é de grande preocupação, já que ainda não se estabeleceu um protocolo medicamentoso definitivo. O uso de corticóides sistêmicos não se mostrou eficaz na diminuição da infiltração leucocitária polimorfonuclear ou do tamanho da lesão em experimentos controlados em animais. Com exceção do abrandamento do envenenamento

sistêmico, eles provavelmente não têm função terapêutica. Além do mais, a injeção de tais fármacos, no local da lesão, pode aumentar o edema e a pressão no tecido lesionado contribuindo para a necrose tecidual (FUTRELL, 1992).

Com base em dados anteriores, pode-se dizer que a possibilidade de uso do soro antiveneno é de pouca função terapêutica, sendo que os indivíduos picados, em geral, só procuram tratamento médico 24 horas após o acidente loxoscélico. É sabido que o veneno loxoscélico pode ficar retido no local da picada por um período de tempo, particularmente em região de tecido gorduroso. Assim, se o antiveneno tem um papel terapêutico, poderia ser não o de prevenção do aparecimento da lesão, mas de remoção do veneno residual, favorecendo a cicatrização periférica da picada.

Nesse sentido, em abril de 1986, KING E REES recomendaram um protocolo a ser seguido para tratamento de picadas severas de aracnídeos e correlacionados, sendo a primeira medida terapêutica a administração dos antibióticos eritromicina ou cefalosporina. Ainda recomendaram: (1) dapsona (se excluída deficiência em G6PD); (2) gelo e elevação do local; (3) evitar atividade exagerada; (4) evitar calor e cirurgia imediata e (5) aspirina. Além dessas medidas, é recomendada a realização de exames laboratoriais adicionais para se tentar identificar qualquer sinal de complicações sistêmicas (FUTRELL, 1992). Ensaios experimentais em coelhos indicam que pode ser útil a administração de antibióticos do grupo da penicilina G, para se evitar a proliferação de microorganismos associados à lesão, como por exemplo, *Clostridium perfringens* (MONTEIRO *et al.,* 2002).

1.5 Características do veneno de aranhas do gênero *Loxosceles*

O veneno loxoscélico é um líquido cristalino, que consiste essencialmente de proteínas, produzido por glândulas situadas no cefalotórax do animal, que se comunicam com o exterior através do aparelho inoculador constituído por um par de quelíceras (GAJARDO-TOBAR, 1966; MARTINEZ-VARGAS, 1987). A quantidade de veneno seco obtida varia entre as espécies. Enquanto animais da espécie *L. rufipes* secretam, em média, 700μg de proteínas por extração (LUCAS, 1988); uma aranha adulta da espécie *L. reclusa* libera no volume total de veneno (aproximadamente 4μl) de 65 a

100mg de proteína (FORRESTER *et al*., 1978). O veneno é uma mistura de toxinas com predominância em proteínas de baixa massa molecular (5-40kDa) (MOTA E BARBARO, 1995; DA SILVEIRA *et al.*, 2002; DA SILVA *et al.*, 2004).

O veneno da aranha marrom pode ser comparado a alguns venenos de cobras, onde diferentes estudos demonstraram a presença abundante de enzimas em sua composição (MARTINEZ-VARGAS, 1987; DA SILVEIRA *et al.*, 2002). No entanto, o conteúdo total do veneno ainda não é totalmente conhecido, além da grande quantidade de proteínas, sabe-se que também contém ácidos nucléicos, aminoácidos livres, poliaminas neurotóxicas, monoaminas e sais inorgânicos. Sabe-se que a toxicidade do veneno de aranhas é decorrente do efeito combinado (efeitos tóxicos sinérgicos) de todos os seus componentes (GEREN *et al*., 1976).

Os mecanismos pelos quais o veneno de aranhas do gênero *Loxosceles* causam a lesão dermonecrótica e/ou disfunção renal estão sob investigação. Várias toxinas têm sido identificadas e bem caracterizadas nos venenos loxoscélicos, entre as quais pode-se destacar fosfatase alcalina, fosfohidrolase ribonucleotidídica, hialuronidase, serino-proteases, metaloproteases e esfingomielinase-D (FUTRELL, 1992; FEITOSA *et al.*, 1998; VEIGA *et al.*, 2000a; DA SILVA *et al.*, 2004).

Metaloproteases denominadas Loxolisina A (20-28kDa) and Loxolisina B (32-35kDa) possuem atividades gelatinolítica, fibronectinolítica e fibrinogenolítica, e consequentemente podem exercer um papel importante nos distúrbios hemostáticos descritos após o envenenamento. Tais como os danos observados nos vasos sanguíneos, hemorragia da derme, disfunção na adesão plaquetária e dificuldade de cicatrização (FEITOSA *et al.*, 1998; DA SILVEIRA *et al*., 2002; ZANETTI *et al.*, 2002).

A toxina hialuronidase degrada ácido hialurônico e resíduos de condroitin sulfato de proteoglicanos e provavelmente poderiam estar envolvidas no espalhamento gravitacional da lesão dermonecrótica e como um fator de espalhamento sistêmico (FUTRELL, 1992; YOUNG E PINCUS, 2001; DA SILVA *et al.*, 2004).

A esfingomielinase-D (30-35kDa) também nomeada toxina dermonecrótica é a molécula melhor caracterizada no veneno de diferentes espécies de *Loxosceles* (RAMOS-CERRILLO *et al.,* 2004; BARBARO *et al*., 2005; ANDRADE *et al*., 2005). Tal toxina como molécula nativa ou em

variantes recombinantes pode induzir dermonecrose, agregação plaquetária e hemólise experimental (KURPIEWSKI *et al.*, 1981; TAMBOURGI *et al.*, 1998a; KALAPOTHAKIS *et al.*, 2002; PEDROSA *et al.*, 2002; CUNHA *et al.*, 2003).

O efeito citotóxico direto ou indireto (p.ex., degradação de componentes da matriz extracelular, VEIGA *et al*., 2001b) tem sido descrito sobre diferentes tipos celulares, destacando linhagens celulares endoteliais (PATEL *et al*., 1994; VEIGA *et al.*, 2001a). Efeito hemolítico direto sobre eritrócitos e ação sobre plaquetas causando sua agregação (FUTRELL, 1992; WILLIAMS *et al.*, 1995), efeito inibitório direto na quimiotaxia de neutrófilos *in vitro* (MAJESTIK *et al.*, 1977), no entanto, por outro lado, induz indiretamente uma intensa ativação de neutrófilos dependente do endotélio (PATEL *et al.*, 1994), a qual parece estar ligada com o desenvolvimento da lesão dermonecrótica (FUTRELL, 1992; DA SILVA *et al.*, 2004). Esta última hipótese foi reforçada pelos achados histopatológicos em coelhos experimentalmente expostos ao veneno e em biópsias de pacientes acidentados (YIANNIAS E WINKELMANN, 1992; ELSTON *et al.*, 2000; OSPEDAL *et al.*, 2002; DA SILVA *et al.*, 2004).

Coutinho (1996) relatou alterações histopatológicas importantes da integridade renal em ratos que receberam doses subletais de veneno de aranha marrom, no entanto, acredita que o efeito observado foi decorrente, principalmente, da deposição de hemoglobina livre ao longo dos néfrons, o que retardaria o fluxo tubular e da filtração glomerular, induzindo a IRA. Os distúrbios renais provocados pelo veneno de aranha marrom têm sido apenas descritos, na maioria dos trabalhos, por dados clínicos de acidentados (FUTRELL, 1992; LUNG e MALLORY, 2000; DA SILVA *et al.*, 2004). O presente trabalho, com intuito de contribuir no entendimento do mecanismo pelo qual o veneno de aranha marrom induz as alterações descritas, pretende avaliar a atividade citotóxica direta *in vivo* e *in vitro*, destacando o efeito nefrotóxico observado nos envenenamentos.

2. OBJETIVOS

- Produção e obtenção de anticorpos que reconheçam toxinas presentes no veneno de aranha marrom (gênero *Loxosceles*).
- Utilização de anticorpos na análise de diferentes atividades biológicas das toxinas loxoscélicas.
- Imunomarcação em tecidos e modelos celulares em cultivo expostos ao veneno de aranha marrom (gênero *Loxosceles*).
- Análise da atividade citotóxica do veneno de aranha marrom sobre diferentes linhagens celulares.
- Análise da atividade nefrotóxica do veneno de aranha marrom.
- Analise do efeito nefrotóxico da toxina dermonecrótica recombinante *in vivo* e *in vitro.*

3. RESULTADOS (ARTIGOS)

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ARTICLE

Experimental Evidence for a Direct Cytotoxicity of *Loxosceles intermedia* (Brown Spider) Venom in Renal Tissue

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SUMMARY Brown spider (*Loxosceles* genus) venom causes necrotic lesions often accompanied by fever, hemolysis, thrombocytopenia, and acute renal failure. Using mice exposed to *Loxosceles intermedia* venom, we aimed to show whether the venom directly induces renal damage. The experimental groups were composed of 50 mice as controls and 50 mice that received the venom. Light microscopic analysis of renal biopsy specimens showed alterations including hyalinization of proximal and distal tubules, erythrocytes in Bowman's space, glomerular collapse, tubule epithelial cell blebs and vacuoles, interstitial edema, and deposition of eosinophilic material in the tubule lumen. Electron microscopic findings indicated changes including glomerular epithelial and endothelial cell cytotoxicity as well as disorders of the basement membrane. Tubule alterations include epithelial cell cytotoxicity with cytoplasmic membrane blebs, mitochondrial changes, increase in smooth endoplasmic reticulum, presence of autophagosomes, and deposits of amorphous material in the tubules. We also found that the venom caused azotemia with elevation of blood urea levels but did not decrease C3 complement concentration or cause hemolysis in vivo. Confocal microscopy with antibodies against venom proteins showed direct binding of toxins to renal structures, confirmed by competition assays. Double-staining immunofluorescence reactions with antibodies against type IV collagen or laminin, antibodies to venom toxins, and fluorescent cytochemistry with DAPI revealed deposition of toxins in glomerular and tubule epithelial cells and in renal basement membranes. Two-dimensional electrophoresis showed venom rich in low molecular mass and cationic toxins. By immunoblotting with antibodies to venom toxins on renal extracts from venom-treated mice, we detected a renal binding toxin at 30 kD. The data provide experimental evidence that *L. intermedia* venom is directly involved in nephrotoxicity. **(J Histochem Cytochem 52:455–467, 2004)**

KEY WORDS
brown spider
venom
toxins
nephrotoxicity

Loxoscelism, the term used to describe lesions and reactions induced by bites from spiders of the genus *Loxosceles*, causes necrosis and local hemorrhage at the bite site, platelet aggregation, thrombocytopenia, disseminated intravascular coagulation, hemolysis, and renal failure at the systemic level (Futrell 1992; Elston et al. 2000; Lung and Mallory 2000; Veiga et al. 2001b; Zanetti et al. 2002). Symptoms such as weakness, fever, nausea, vomiting, and convulsions, which suggest some effect on the nervous system, have also been reported (Futrell 1992).

A number of enzymes and biologically active molecules that might contribute to the deleterious effects of the venom have been identified and biochemically characterized. A sphingomyelinase D of 32–35 kD isolated from brown spider venom can induce dermonecrosis, platelet aggregation, and experimental hemolysis (Futrell 1992). Metalloproteases of 32–35 kD and

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20–28 kD identified in the venom with gelatinolytic, fibronectinolytic, and fibrinogenolytic activities can also play a role in hemorrhage evoked by envenomation, such as hemorrhage into the dermis, injury of blood vessels, imperfect platelet adhesion, and the defective wound healing observed in some cases (Feitosa et al. 1998; Veiga et al. 2001a,b; da Silveira et al. 2002; Zanetti et al. 2002). A hyaluronidase with electrophoretic mobilities at regions of 33 kD and 63 kD is likely to contribute to the gravitational spread of dermonecrotic lesions, a hallmark of brown spider bites (Wright et al. 1973; Futrell 1992). A number of other molecules and activities have been identified in the venom, including a lipase, alkaline phosphatase, and proteolytic activities on entactin, basement membranes, and the protein core of a heparan sulfate proteoglycan from endothelial cells (Futrell 1992; Veiga et al. 2000,2001b). The mechanism underlying the involvement of these individual venom constituents or activities in the noxious effects of the venom on cell tissue has not been fully determined.

Some reports have indicated the participation of blood cells and molecular components in the noxious effects of the venom. The serum amyloid P component appears to be a target for platelet activation and ischemic effects and is likely to play a role in the necrosis caused by the venom (Gates and Rees 1990). Leukocytes, and especially polymorphonuclear cells (PMNs) such as neutrophils and eosinophils, appear to play a role in the dermonecrotic lesion evoked by the venom, because histopathological findings have revealed a massive infiltration of these cells into the dermis and related structures in the dermonecrotic regions induced by the venom (Elston et al. 2000; Ospedal et al. 2002). Depletion of leukocytes in the blood results in reduction of clinical signs in the skin injected with venom (Smith and Micks 1970). The complement system in the plasma also appears to participate in the deleterious activities of the venom, especially on erythrocytes, evoking a complement-dependent hemolysis (Futrell 1992).

Although some clinical signs of loxoscelism have been well described, and putative molecules in the venom and physiopathological events involved in cell destruction have been characterized as described above, data about renal disorders evoked by brown spiders have been limited to earlier reports describing clinical data from victims (Futrell 1992; Lung and Mallory 2000). We report here the effect of *L. intermedia* venom on kidney structures. Mice were used because these animals do not develop dermonecrotic lesions induced by *Loxosceles* venom, so that the occurrence of nephrotoxicity secondary to complications of dermonecrotic lesions can be ruled out. We hope to bring some insight into loxoscelism that could be useful to physicians who diagnose and treat the victims.

Materials and Methods

Reagents

Polyclonal antibodies to *L. intermedia* venom toxins were produced in a rabbit using crude venom as antigen and complete Freund's adjuvant (Sigma; St Louis, MO) for the first primary injection at one SC point and two IM points (20 μg of proteins as total antigens were used divided into three parts). As a booster for the second, third, and fourth injections, incomplete Freund's adjuvant (Sigma) was similarly injected at 2-week intervals. Twelve days after the last immunization, blood was collected and the production of specific antibodies analyzed by ELISA and Western blotting (Harlow and Lane 1988). Hyperimmune IgGs were purified from serum using protein–A Sepharose (Amersham Biosciences; Piscataway, NJ) as recommended by the manufacturer. Polyclonal antibodies that recognize type IV collagen were purchased from Chemicon International (Temecula, CA) and monoclonal antibodies against laminin were purchased from DAKO (Carpinteria, CA). Fluorescein- and rhodamine-conjugated anti-IgG antibodies were purchased from Chemicon. DAPI (4′,6-diamidino-2-phenylindole HCl) nucleic acid stain was purchased from Molecular Probes (Eugene, OR).

Spider Venom Extraction

The venom was extracted from spiders captured from the wild and kept for a week without any food but with water ad libitum. The venom was extracted from the fangs of spiders by electrostimulation (15 V) applied to the cephalothorax and was collected with a micropipette, dried under vacuum, and frozen at −85C until use. Pools of venom collected from 100 to 200 spiders in different batches were used during all the experiments, involving approximately 1000 spiders (Feitosa et al. 1998). Protein content was determined by the Coomassie Blue method (Bradford 1976).

Animals

Adult Swiss mice weighing approximately 25 g from the Central Animal House of the Federal University of Paraná were used for in vivo experiments with the venom. All experimental protocols using animals were performed according to the "Principles of Laboratory Animal Care" (NIH Publication 85-23, revised 1985) and Brazilian federal laws.

Venom Administration

Pooled crude venom and mouse samples of 1 mg of protein/kg were diluted in PBS (pH 7.3). These samples were injected IP in a volume of 100 μl in each mouse. The animals were divided into two groups, a control (C) group and a test (T) group. The control group consisted of five animals receiving only PBS and the test group consisted of five animals receiving *L. intermedia* venom. During the experimental procedures, the envenomation of animals was repeated at least 10 times, completing a number of 50 animals as controls and 50 animals that received the venom. All animals were kept under the same experimental conditions. All kidney and blood samples were collected from living animals.

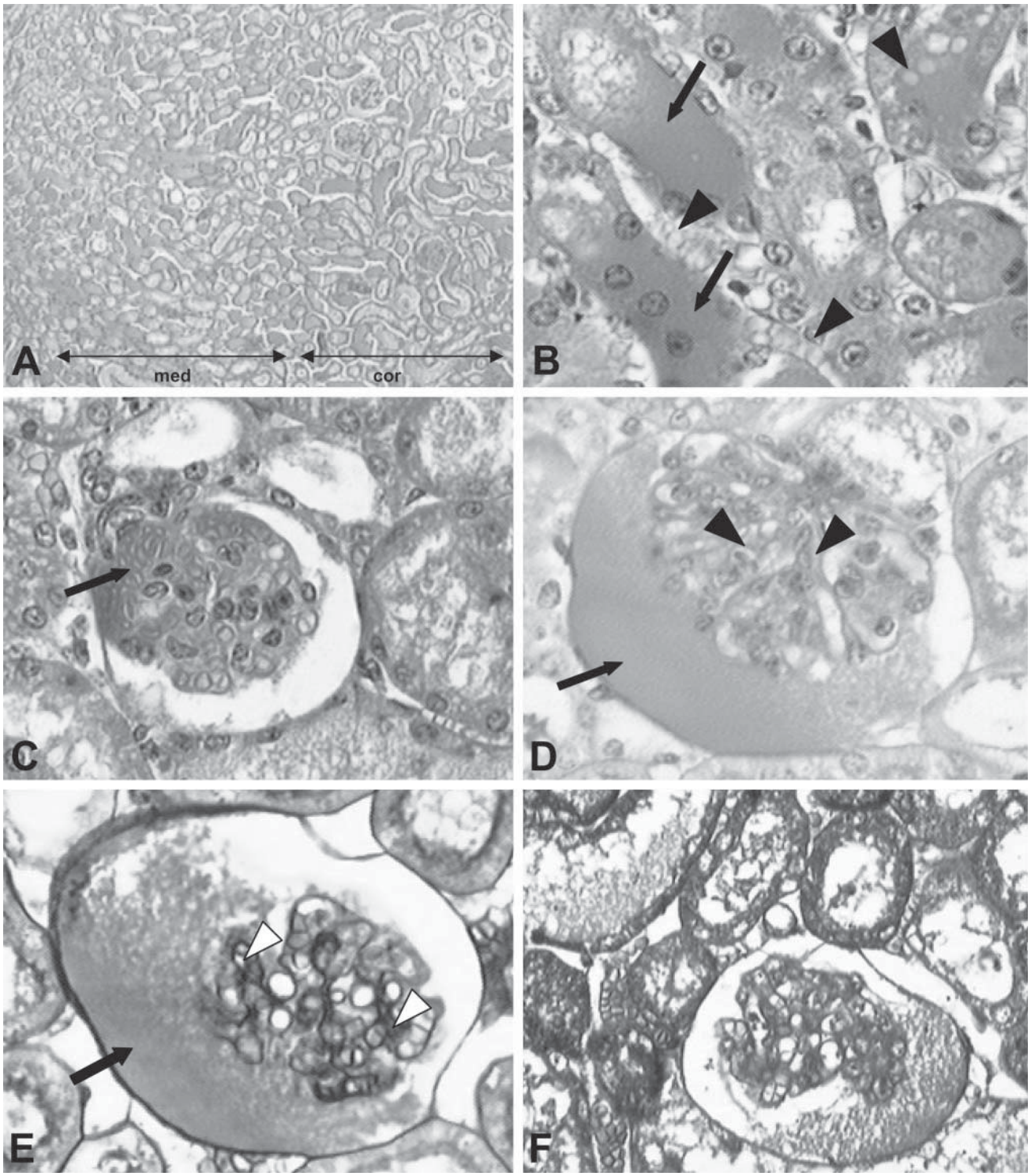

Figure 1 Light microscopic analysis of kidneys from venom-treated mice. Sections of kidneys from mice treated with *L. intermedia* venom were stained with a combination of dyes and analyzed by light microscopy. (**A**) Panoramic view of a kidney stained with hematoxylin and eosin, indicating diffuse alterations along medullary (med) and cortical (cor) regions. Magnification ×40. (**B**) Details of tubule structures stained with hematoxylin and eosin, showing accumulation of eosinophilic material within the tubule lumen (arrows) and vacuoles in the epithelial cells (arrowheads). (**C**) Inset of a cross-section of glomerular structures stained with hematoxylin and eosin, where focal intraglomerular erythrocytes can be seen in Bowman's space (arrow). (**D,E**) Glomerular cross-sections stained with periodic acid Schiff reagent plus hematoxylin and silver impregnation, where collapse of basement membranes (arrowheads) and deposition of proteinaceous materials in Bowman's space (arrows) can be observed. (**F**) Cross-section of glomerular and tubule structures stained by the Rosenfeld method. No leukocyte infiltration can be observed. Magnifications ×400.

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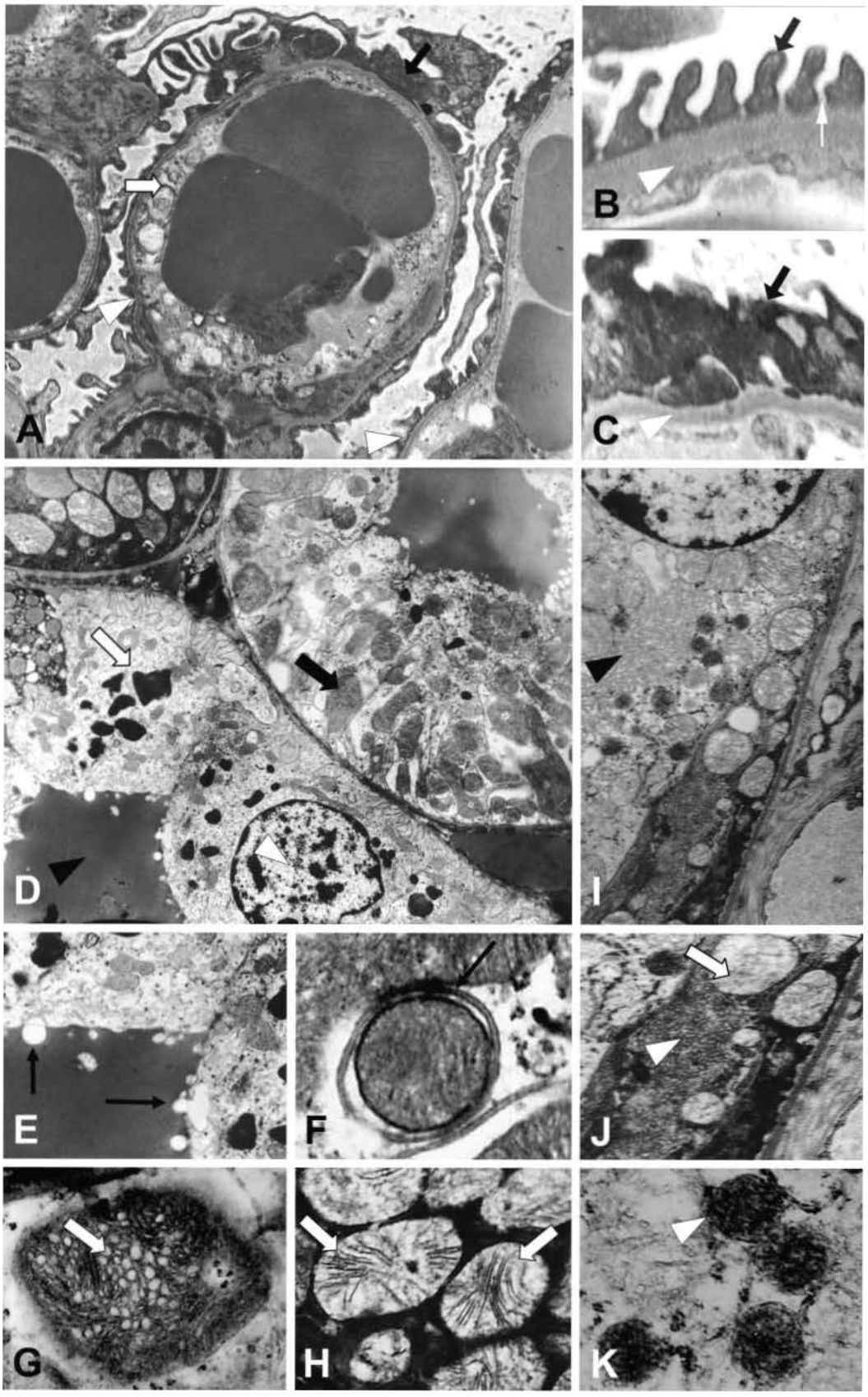

Kidney and Blood Sample Collections and Laboratory Analysis

Kidney and blood (directly from the heart) samples were obtained from mice anesthetized with ketamine (Agribands; Paulinia, SP, Brazil) and acepromazin (Univet; São Paulo, SP, Brazil). Blood was anticoagulated with EDTA-K3 and used for red cell, hemoglobin, hematocrit, leukocyte, and platelet counts that were determined with an automated CELL-DYN 1,400 blood counter (Abbott Laboratories; Chicago, IL). Urea and C3 complement were determined in serum. Assays were performed using standardized techniques and reagents as described by Kaplan and Pesce (1996) and Henry (2001).

Statistical Analysis

The Student's *t*-test for unpaired observations was used to detect statistically significant differences between control and test groups for red cell concentration (10^6 ml/blood), hemoglobin (g/dl blood), hematocrit (%), platelets (10^3 ml/blood), leukocytes (10^3 ml/blood), serum urea (mg/dl), and C3 complement (mg/dl). The threshold level for significance was $P=0.05$. All statistical calculations were done with the GraphPad InStat program version 3.00 for Windows 95. Morphometric analysis of sections stained with hematoxylin and eosin or from electronmicrographs were measured by use of software Jandel Sigma Scan Pro.

Gel Electrophoresis

Lysed renal cells were obtained by treatment of kidneys with lysis buffer (50 mM Tris-HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 1 mM $CaCl_2$,1 mM phenylmethanesulfonyl fluoride, and 2 μg/ml aprotinin) for 15 min at 4C. The extract was clarified by centrifugation for 10 min at 13,000 × g. Renal extracts or crude venom (normalized for their protein contents) were submitted to electrophoresis under non-reducing conditions. Linear gradient 3–20% or 8–18% SDS-PAGE was performed as described by Laemmli (1970). For protein detection, gels were stained with Coomassie Blue. For immunoblotting, proteins were transferred to nitrocellulose filters overnight as described by Towbin et al. (1979). The molecular mass markers used were from Sigma. A two-dimensional gel was run with some modifications as previously described by the manufacturers using Immobiline Dry Strip Gel, pH range 3–10 (Amersham Biosciences). Crude venom 100 μg collected in water was diluted in rehydration solution (6 M urea, 2 M thiourea, 2% w/v CHAPS, 1% IPG buffer, and a trace of bromophenol blue) and applied to an IEF strip. The second dimension was carried out using 8–18% linear gradient SDS-PAGE under non-reducing conditions. Gel was stained with the silver method as described by Heukeshoven and Dernick (1986).

Histological Methods for Light Microscopy

Kidneys were fixed in modified Carnoy's fixative (5% acetic acid instead of 10% as originally proposed) for 3 hr. After fixation, tissues were processed for histology, embedded in paraffin, and cut into 4-μm sections. The sections were stained with hematoxylin and eosin, acid-Schiff (PAS), and silver and by the method of Rosenfeld (Culling et al. 1985; Beautler et al. 1995).

Transmission Electron Microscopy

Kidneys were fixed with modified Karnovsky's fixative (without calcium chloride and with glutaraldehyde 2.5%) (Karnovsky 1965) for 2 hr, washed in 0.1 M cacodylic acid buffer, pH 7.3, postfixed in 1% OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 hr, dehydrated with ethanol and propylene oxide, embedded in Epon 812, contrasted with uranyl acetate and lead citrate, and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV (Peabody, MA).

Immunofluorescence and Fluorescence Cytochemistry

For immunofluorescence microscopy, kidney tissues were fixed with 2% formaldehyde in PBS for 30 min at 4C, incubated with 0.1 M glycine for 3 min, and blocked with PBS containing 1% BSA for 1 hr at room temperature (RT). Histological sections were incubated for 1hr with specific antibodies raised against laminin (0.33 μg/ml), type IV collagen (1:40), and venom toxins (2.0 μg/ml) as described above. The sections were washed three times with PBS, blocked with PBS containing 1% BSA for 30 min at RT, and incubated with fluorescein- or rhodamine-conjugated anti-IgG secondary antibodies (Chemicon) at RT for 40 min. After washing with PBS, samples were observed under a confocal

Figure 2 Ultrastructural findings of kidneys from venom-treated mice. Transmission electron micrographs of cross-sectioned kidneys exposed to the venom. **(A)** General view of glomerular structures showing alterations of foot processes (black arrow), basement membrane (white arrowhead), and capillary endothelial cells (white arrow). Magnification ×10,000. Comparison of glomerular filter structure between non-injected **(B)** and venom-treated mice **(C)** shows loss of foot processes (black arrows), alteration of filtration slits (white arrow), and fenestrated endothelium as well as reduced thickness of the basement membrane (white arrowheads). Magnification ×20,000. **(D)** Analysis of tubule epithelial cells shows signs of damage such as increased numbers of cytoplasmic electron-dense bodies (white arrow), mitochondrial injuries (black arrow), and deposition of amorphous substances in the tubule lumen (black arrowhead), although the nucleus is preserved (white arrowhead). Magnification ×10,000. **(E)** Details of tubule epithelial cells show blebs in the cytoplasmic membrane (arrows), providing further evidence of cell necrosis. Magnification ×12,000. **(F)** The enclosure of an organelle originating an autophagosome (arrow). Magnification ×15,000. **(G,H)** Details showing deranged and vacuolated mitochondria (arrows). Magnification ×20,000. **(I)** Details of the cytoplasm of a tubule epithelial cell where abundant smooth endoplasmic reticulum can be observed (arrowhead). Magnification ×12,000. **(J)** Again, another sign of cell damage shown by increased smooth endoplasmic reticulum (arrowhead) and an autophagosome of a damaged organelle (arrow). Magnification ×15,000. **(K)** Accumulation of electron-dense bodies in the cell cytoplasm (arrowhead). Magnification ×15,000.

fluorescence microscope (Confocal Radiance 2,100; BioRad, Hercules, CA) coupled to a Nikon–Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division; Melville, NY). For nuclear fluorescence cytochemistry, samples of renal tissue were incubated with DAPI (O.5 μg/mL diluted in PBS) for 5 min (Molecular Probes). The samples were washed and observed under a confocal microscope as above. For antigen competition assay, the immunofluorescence protocol was the same as described above except that the hyperimmune serum to venom toxins was incubated previously for 1 hr with 50 μg/ml of crude venom diluted in PBS. Then the mixture was incubated with renal biopsies identically as above.

Results

Histopathological Findings in Kidneys from Mice That Received *L. intermedia* Venom

To obtain information about the degree of renal damage caused by *Loxosceles* venom, mice were exposed IP to *L. intermedia* venom for 4 hr. As shown in Figure 1, the effects of the venom on the kidney revealed a complex pattern of nephrotoxicity. Light microscopy and histochemistry techniques revealed glomerular alterations consisting of collapse of basement membranes and deposition of hyaline and eosinophilic masses in the glomeruli (hyalinization), as well as focal intraglomerular erythrocytes. Tubule alterations were detected by accumulation of eosinophilic material in the lumen of cortical and medullary tubules, interstitial edema with extravascular red blood cells, and vacuolar degeneration of proximal and distal tubules. Morphometric analysis (Figure 1A) showed that 75–80% of the kidney area was affected by the venom. No glomerular or tubule leukocyte infiltration was detected.

Ultrastructural Evidence of Renal Injuries

To improve the evidence of the activity of brown spider venom toxin on kidney structures and to better understand these alterations, biopsies from venom-treated mice were studied by transmission electron microscopy. As shown in Figure 2, additional evidence of glomerular damage was provided by structural signs of podocyte cytotoxicity as shown by many disturbed foot processes (pedicels) and filtration slits. At the capillary level, fenestrated endothelial cells also showed signs of alterations, such as detachment from subendothelial basement membrane and structural signs of disorganization of the fenestra. The basement membrane was decreased compared to the control group. Morphometric analysis of glomerular basement membranes showed a general reduction of approximately 24%. Tubule injuries included deposition of amorphous electron-dense material in the distal tubule lumen. Necrotic destruction of tubule epithelial cells can be visualized by vacuolization and blebs on the cytoplasmic membrane, diffuse destruction of mitochondria, prominent smooth endoplasmic reticulum, and autophagosomes indicating necrotic cell destruction.

Table 1 Blood counts[a,b]

	WBC ($10^3/\mu l$)	RBC ($10^6/\mu l$)	HGB (g/dl)	HCT (%)	PLT ($10^3/\mu l$)[c]
Controls					
C1	5.0	9.6	15.7	47.4	682
C2	5.3	7.2	12.4	36.2	869
C3	3.0	7.7	12.9	35.7	818
C4	6.5	8.1	13.2	37.3	652
C5	4.1	8.0	14.2	40.2	900
Venom					
V1	5.3	8.5	13.3	36.7	265
V2	7.5	8.2	13.7	41.2	395
V3	3.8	8.1	13.0	39.8	261
V4	6.2	7.8	12.5	39.2	244
V5	6.6	8.0	12.5	38.6	418

[a]WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets.
[b]Significance is defined as $p<0.05$, Student's *t*-test.
[c]PLT significant differences ($p<0.0001$).

Laboratory Investigations After Administration of Brown Spider Venom

Our initial approach to study the involvement of venom-induced hemolysis in renal pathogenesis in the experimental model used was to determine blood cell counts, urinalysis, C3 complement, and serum urea and to compare venom-treated mice with a control group. As shown in Table 1, neither red blood cell count, hemoglobin, or hematocrit values showed significant differences between venom-treated and control groups, suggesting that the venom was unable to lyse mouse erythrocytes in vivo under the conditions assayed. In addition, no significant evidence of hemoglobinuria or of the presence of red blood cells in urine was observed in venom-treated mice (Table 2), indicating that hemolysis was not taking place. Table

Table 2 Urinalysis[a]

	Hemoglobinuria	Hematuria
Controls		
C1	–	–
C2	–	–
C3	–	–
C4	–	–
C5	–	–
Venom		
V1	–	+
V2	–	–
V3	–	–
V4	+	–
V5	–	–

[a]–, negative; +, trace.

Table 3 Complement (C_3)

	C_3 concentration (mg/dl)[a]
Controls	
C1	21.8
C2	16.7
C3	20.6
C4	20.5
C5	23.9
Venom	
V1	19.6
V2	16.6
V3	26.3
V4	19.0
V5	17.3

[a]Significance is defined as $p<0.05$, Student's *t*-test.

3 shows no significant differences in C3 complement component between venom-treated and control mice, demonstrating that the venom was unable to decrease this serum molecule in vivo under the conditions used. On the other hand, as shown in Table 4, serum urea was significantly increased in venom-treated mice compared to control group. Finally, renal biopsies from mice treated with the brown spider venom were negative for iron when submitted to histochemistry by the Prussian blue method (Figure 3), supporting the idea that venom-induced hemolysis was not involved in nephrotoxicity in the model used.

Evidence That *Loxosceles* Venom Toxins Bind Directly to Kidney Structures

To study the molecular pathways of venom pathogenesis in the kidney, we investigated the possibility of a direct interaction of venom toxins with kidney structures by submitting renal biopsies from venom-treated and control mice to immunofluorescence using affinity-purified hyperimmune IgG that reacts with venom toxins (Montero 2003). As shown in Figure 4, we

Table 4 Urea[a]

	Urea concentration[b] (mg/dl)
Controls	
C1	41.0
C2	37.0
C3	42.0
C4	36.0
C5	46.0
Venom	
V1	60.0
V2	59.0
V3	63.0
V4	70.0
V5	46.0

[a]Significance is defined as $p< 0.05$, Student's *t*-test.
[b]Significant differences ($p=0.0021$).

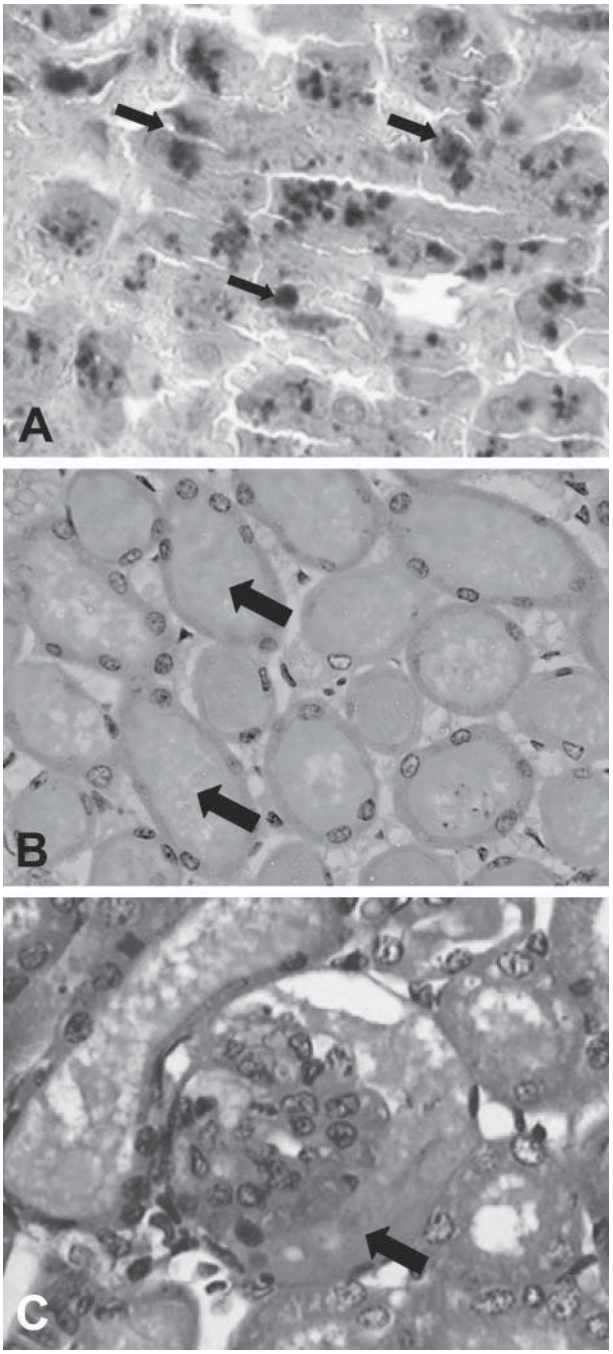

Figure 3 Sections from venom-treated kidneys stained with Prussian blue. *L. intermedia* venom-treated sections from mouse kidneys were stained with Prussian blue plus hematoxylin to detect iron (hemoglobin) deposition along the renal structures. Although deposition of proteinaceous materials in the tubule lumen (arrows) (**B**) and glomerular Bowman's space (arrow) (**C**) can be seen, the results were negative for iron (hemoglobin) because there was no precipitated blue material as found in the positive control group by using a liver section from a normal rabbit (arrows) (**A**). Magnification ×400.

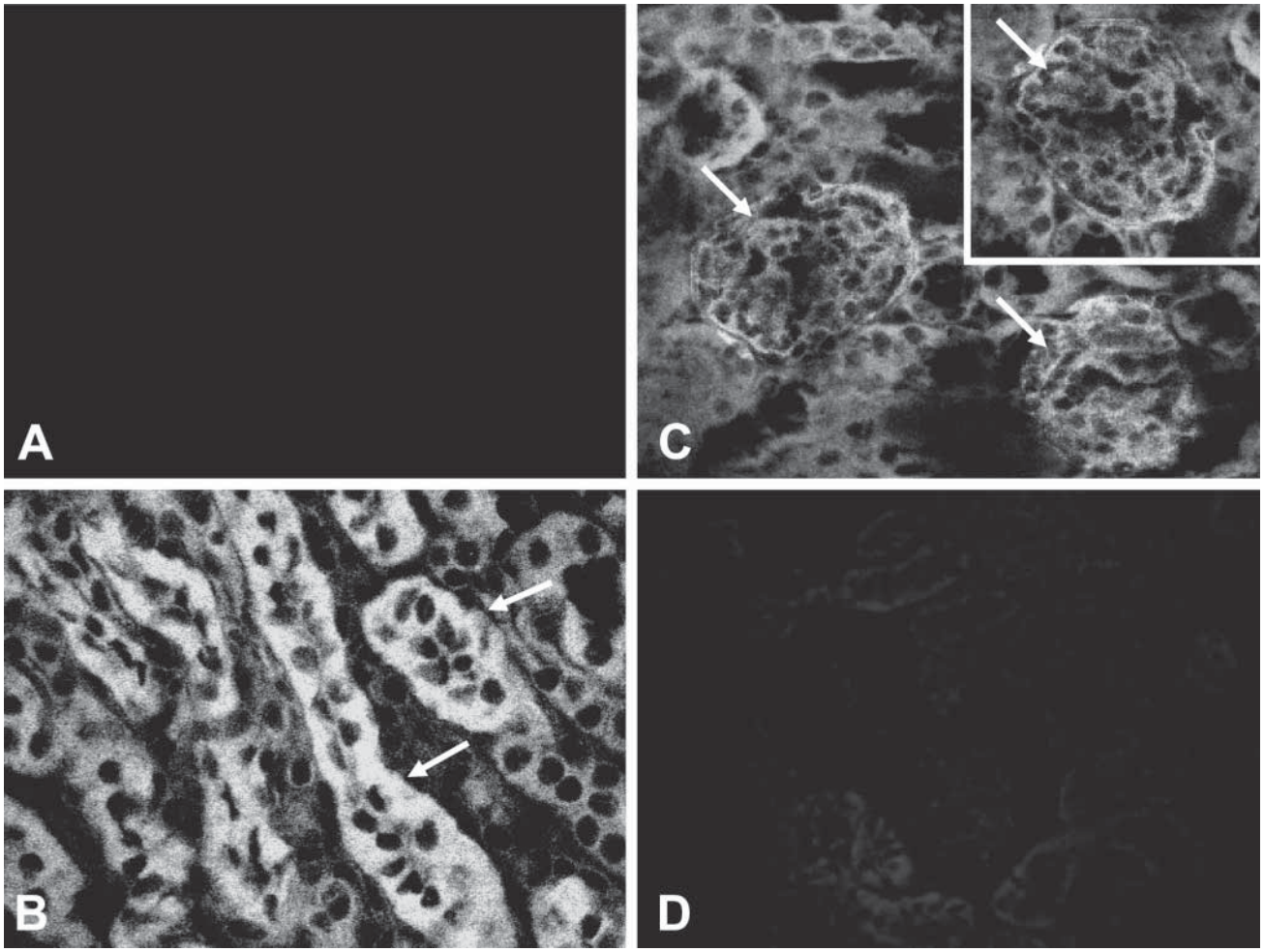

Figure 4 Confocal immunofluorescence microscopy analysis of kidney sections from venom-treated mice. Cross-sectioned kidneys immunolabeled with purified antibodies against *L. intermedia* venom toxins. (**A**) Section of a kidney from the control group that did not receive the venom. (**B,C**) Kidney sections from venom-treated mice, respectively showing regions rich in tubules and glomeruli (arrows) positive for binding of toxin antibodies. (**D**) Result of an antigen competition assay in which antibodies to venom toxins were previously incubated with crude venom in solution. A kidney section from a venom-treated mouse was exposed to the reaction mixture under conditions identical to those described above. The results indicated a highly representative decrease in immunolabeling and confirmed toxins as "planted antigens" in kidney structures from venom-treated mice. Magnifications ×200.

found that hyperimmune IgG produced a positive reaction in renal biopsies from venom-treated mice but did not react with biopsies from normal mice by immunofluorescence. In addition, confirm the antibody-specific interactions of venom toxins with the kidney, we repeated the same immunofluorescence approach, this time incubating hyperimmune IgG with a medium containing venom in solution and then exposing renal biopsies from venom-treated mice to this mixture (antigen competition assay). We found that soluble venom effectively inhibited binding of antibodies to the kidney, supporting the evidence of venom toxins as "planted antigens" bound to renal structures.

Renal Injuries by *Loxosceles* Venom Resulting from Binding of Toxins to Glomerular and Tubule Cells and Basement Membrane Structures

After confirming the direct binding of venom toxins to the kidney, we examined the possibility that intrinsic kidney components were targets of the toxins. We speculated that toxins can act as "planted antigens" and bind to renal structures as previously reported for several other antigens such as viral or bacterial products and drugs (Barnes 1989; Kerjaschki and Neale 1996; Cotran et al. 1999). We performed double staining and confocal microscopy immunofluorescence reactions on renal biopsies from venom-treated mice using antibodies that react with type IV collagen and

laminin (two molecular constituents of basement membranes; see Courtoy et al. 1982 and Rohrbach and Timpl 1993) and antibodies that react with venom toxins as well as fluorescent cytochemistry for chromosomes with DAPI. As shown in Figure 5, there was overlapping fluorescence of reactions using antibodies to laminin or type IV collagen and antibodies to venom toxins. Fluorescence staining cytochemistry of nuclei with the blue fluorescent dye DAPI revealed no overlapping with anti-venom fluorescence pattern. These data support diffuse binding and deposition of venom toxins along the glomerular and tubule cells, with prominent staining mainly in tubule cells and along renal basement membranes.

L. intermedia Venom Is Enriched in Cationic and Low Molecular Mass Proteins with Toxin(s) of Approximately 30 kD Binding to Intrinsic Renal Components

To corroborate the findings described above, we looked for the molecules in the venom involved in noxious influences on renal structures. For this purpose and to study venom toxins' physicochemical properties such as their charge and size, crude venom was submitted to a two-dimensional electrophoresis (isoelectric focusing and gradient SDS-PAGE) that was stained by the highly sensitive monochromatric silver method (Figure 6A). The venom was enriched in basic toxins at physiological pH, with low molecular mass in the range of 35–20 kD. Such cationic and low molecular mass properties might explain the localization of venom toxins in the kidney by interacting with components of the nephron. To strengthen such evidence, renal lysates from venom-treated mice were electrophoresed and immunoblotted with hyperimmune serum to venom toxins (see Materials and Methods). Antigen competition assays were also performed to substantiate the specificity of immune reactions. As shown in Figure 6B, we detected a band at 30 kD, in the venom-treated lysate, which specifically reacted with antibodies against venom toxins, confirming the above results and identifying toxin(s) from *L. intermedia* venom as a direct ligand on renal structures.

Discussion

Clinical data have shown that envenomation by brown spiders can have a severe nephrotoxic effect (Futrell 1992; Lung and Mallory 2000). This venom nephrotoxicity, although less common than necrotic lesions and gravitational spreading (which are the hallmark signals of poisoning), is generally involved in the complications that occur after spider bites (Futrell 1992; Lung and Mallory 2000). The clinical and laboratory features observed in victims may include hemoglobinuria and proteinuria (Futrell 1992; Williams et al. 1995; Lung and Mallory 2000). According to some specialists, the pathological processes in the kidney may reflect hematologic disturbances such as intravascular hemolysis and disseminated intravascular coagulation caused by envenomation, which may lead to renal failure (Futrell 1992; Williams et al. 1995; Lung and Mallory 2000). However, there is no direct experimental evidence confirming such a hypothesis. On the other hand, renal disorders may also be the consequence of toxic components of the venom that act directly on renal cells or structures such as glomerular and tubule basement membranes, as demonstrated for several venoms (Bjarnason and Fox 1995; Veiga et al. 2000). Experimentally, it was demonstrated that the venom is cytotoxic to rabbit blood vessel endothelial cells (CLPs) (Veiga et al. 2001b) and human umbilical vein endothelial cells (HUVECs) (Patel et al. 1994) in culture and also to endothelial cells from skin blood vessel walls of rabbits intradermally injected with the venom (Zanetti et al. 2002). The venom also acts on EHS (Engelbreth–Holm–Swarm tumor) basement membrane integrity and on purified basement membrane molecules such as entactin, fibronectin, and heparan sulfate proteoglycan (Feitosa et al. 1998; Veiga et al. 2000,2001b) but, again, there is no direct experimental evidence for venom cytotoxicity to renal cell lines or renal basement membrane structures in vivo.

To obtain additional information about the extent of renal damage and the mechanisms of the disorders induced by *L. intermedia* venom, we presented here laboratory and biochemical data and morphological data from examinations of kidney structures from mice exposed to the venom. Histological studies showed that envenomation induced a complex pattern of nephrotoxicity. A general view of renal tissue revealed alterations at the level of glomerular and tubule structures, supporting the nephrotoxic activity of venom toxins. The glomerular damage becomes apparent by the presence of extravascular red blood cells around the glomerular capillaries in Bowman's space. A protein-rich exudate inside Bowman's space confirms some damage at the glomerular level, suggesting loss of vascular integrity. In addition, tubule injuries are supported by the presence of proteinaceous material in their lumen in addition to tubule edema, causing the tubules to be susceptible to ischemic damage caused by occlusion. Ultrastructural features of nephrotoxicity were further supported by glomerular and tubule disorders. The azotemia detected by the increase in serum urea strengthened this evidence.

The mechanism by which *Loxosceles* venom induces nephrotoxic lesions has remained elusive and is currently unknown. In contrast to cutaneous lesions evoked by the venom, in which leukocytes (neutrophils) play an essential role in pathogenesis (Smith and

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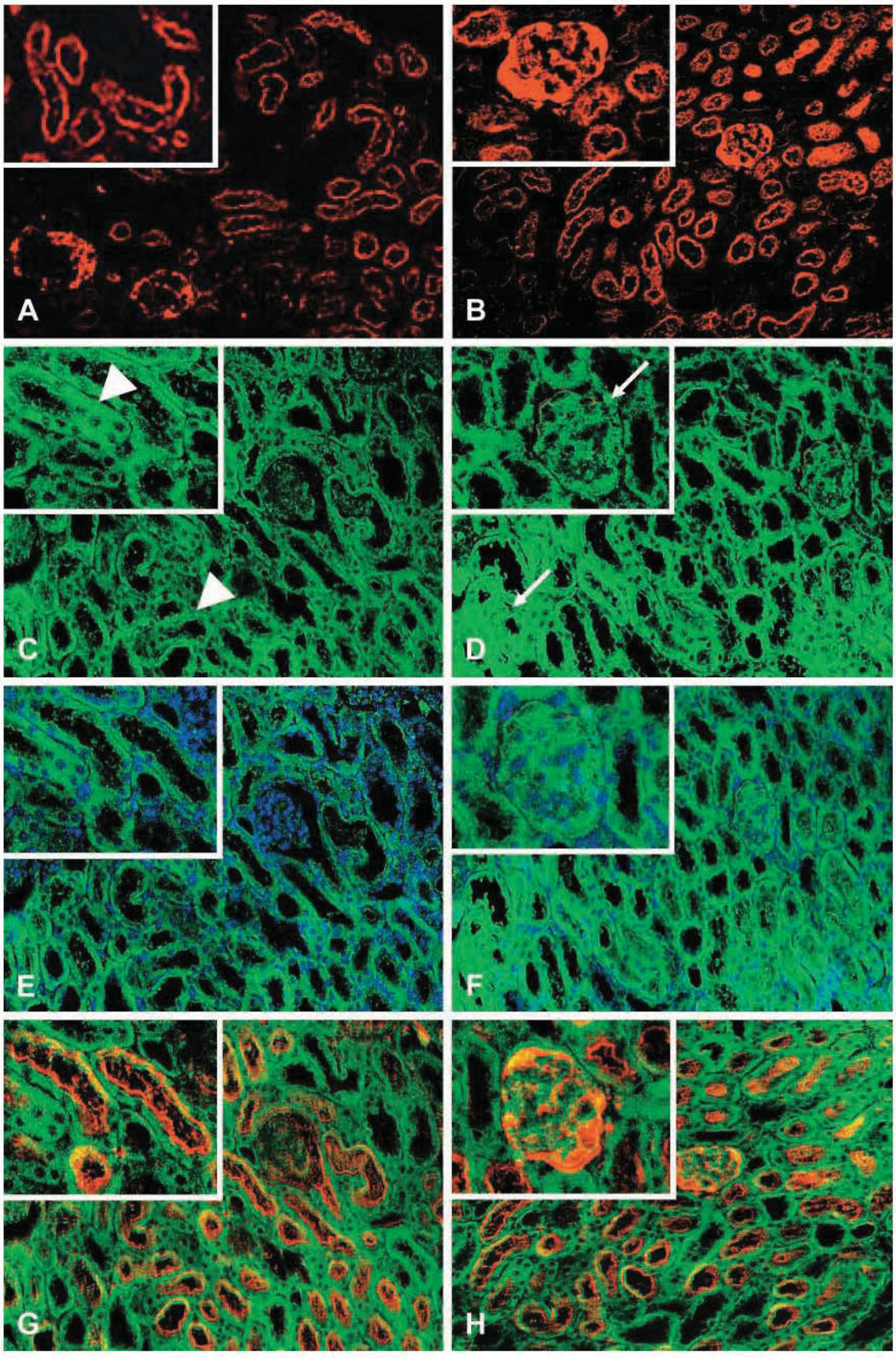

Micks 1970; Futrell 1992; Patel et al. 1994; Ospedal et al. 2002), in the model studied here there was no leukocyte infiltration in the presence of the renal injuries, which were not associated with inflammatory disease.

On the other hand, several case reports have described intravascular hemolysis associated with brown spider envenomation (Murray and Seger 1994; Williams et al. 1995), and many studies have indicated a direct hemolytic activity of *Loxosceles* venom on erythrocytes (Futrell 1992). Such hematological disturbances could be related to renal injuries as secondary agents (Futrell 1992; Williams et al. 1995; Lung and Mallory 2000). We found that, in the model and under the conditions used, despite the renal injuries caused by the venom there was neither a direct hemolytic effect in vivo nor hemoglobin deposition in the kidney structures. These results agree with those described by Futrell (1992), who pointed out the susceptibility of some animal species to venom-induced hemolysis. Human and swine erythrocytes are more susceptible to hemolysis than rabbit and guinea pig erythrocytes. We cannot rule out the involvement of venom hemolysis in the human renal failure evoked by the venom. Nevertheless, because the venom did not induce dermonecrosis (data not shown) or hemolysis in mice and was extremely active against renal integrity, we can speculate about a direct and primary activity of the venom on kidney structures.

We confirmed this possibility by confocal immunofluorescence microscopy using antibodies to venom toxins. We were able to detect toxins as "planted antigens" deposited along the kidney structures of animals exposed to the venom. A competition assay using crude venom toxins in solution blocked the immunofluorescence positivity in the kidney of venom-treated animals, further supporting this evidence and the idea of "planted toxins." The present results agree with several reports indicating the binding of exogenous molecules, such as bacterial products, viral antigens, and drugs, to intrinsic components of renal structures as etiological agents of renal injuries (Kerjaschki and Neale 1996; Cotran et al. 1999). In the model used here, we did not find a role for immune mechanisms in renal injuries. Because the biopsies were collected just 4 hr after exposure to the venom, there was no alteration in C3 complement levels in the serum of venom-treated animals compared to controls, and an immunofluorescence assay with an anti-mouse IgG was negative (data not shown), excluding the possibility of renal deposits of immunoglobulins. Taken together, the above results support the notion that venom toxins act as direct ligands in renal structures and as primary agents, playing a role in renal disorders.

In addition, our data also showed that venom toxins bind to glomerular and tubule cells and basement membranes but do not bind to kidney cell nuclei. This conclusion was based on double-staining immunofluorescence reactions that demonstrated co-localization of venom toxins with basement membrane constituents such as type IV collagen and laminin, but no co-localization along the chromosomes revealed by DAPI. The basement membranes are specialized extracellular matrices involved in several physiological events, especially those dependent on cell adhesion. In the kidney these structures play a role in glomerular filtration during urine formation in addition to organizing podocyte, endothelial, and epithelial cell adhesion (Courtoy et al. 1982; Rohrbach and Timpl 1993; Cotran et al. 1999). The deposition of venom toxins along the renal basement membranes can explain glomerular epithelial cell injury, fenestrated endothelial cell cytotoxicity, hyalinosis, and proteinuria, as well as tubule cell damage. Because some *L. intermedia* venom toxins are proteases, with the ability to degrade basement membrane constituents (as discussed above), such venom injuries can result from loss of renal basement membrane integrity with consequent cytotoxicity to epithelial and endothelial cells and detachment and loss of the glomerular basement membrane charge barrier.

Using two-dimensional electrophoresis, we observed that *L. intermedia* venom is enriched in basic proteins with molecular masses ranging from 35 to 20 kD. Immunoblotting analysis using antibodies to venom proteins identified venom toxin(s) at 30 kD as direct ligands of renal structures. Physicochemical properties

Figure 5 Venom toxin deposition along the glomerular and tubule cells and renal basement membranes detected by confocal double-staining immunofluorescence microscopy or fluorescence cytochemistry. Sections of kidneys from venom-treated mice were incubated with antibodies against basement membrane constituents such as laminin (**A**) or type IV collagen (**B**) and visualized with rhodamine-conjugated antibodies. Similar sections were incubated with antibodies against venom toxins and visualized with fluorescein-conjugated antibodies (**C,D**), respectively (arrows point to glomerular structures and arrowheads show tubule structures). These same sections were cytochemically labeled for nuclei with the blue fluorescent dye DAPI. (**E,F**) Confocal microscopic analysis and overlapping of immunostaining for venom toxins and cytochemistry using DAPI. There was no co-localization of images, supporting the idea that venom toxins do not bind to nuclei of renal cells. On the other hand, overlapping of immunostaining for basement membrane laminin and type IV collagen and immunostaining for venom toxins (**G,H**) respectively, indicated co-localization of toxins with laminin and type IV collagen along glomerular and tubule basement membranes (orange to yellow) as well as glomerular and tubule cells (green). Magnifications ×200; insets ×600).

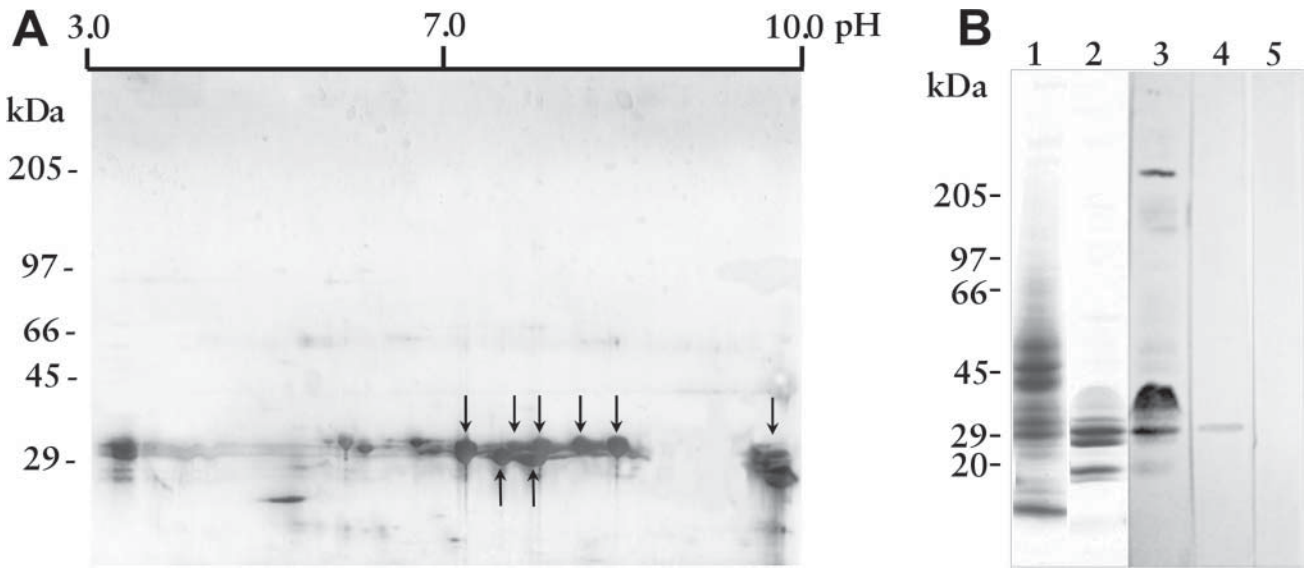

Figure 6 Identification of 30-kD venom toxin(s) as direct ligands of renal structures. Crude *L. intermedia* venom was separated by two-dimensional electrophoresis (isoelectric focusing and linear gradient 8–18% SDS-PAGE) under non-reducing conditions. The gel was stained by the silver method. Molecular mass protein standard positions are shown at left and pH gradient at the top. Arrows point to cationic proteins. (**B**) Renal extract from venom-treated mice (Lanes 1, 4, and 5) or *L. intermedia* Venom (Lanes 2 and 3) was separated by linear gradient 3–20% SDS-PAGE under non-reducing conditions. The gel was stained by the Coomassie Blue method (Lanes 1 and 2) or transferred to a nitrocellulose membrane that was exposed to purified antibodies against venom toxins (Lanes 3 and 4), or antibodies were previously incubated with crude venom in solution and the mixture was exposed to nitrocellulose membrane (Lane 5) in an antigen competition assay. Protein standard masses are shown at left.

such as the molecular charge and size of "planted antigens" along the kidney are very important factors that affect the interaction of these antigens along the renal basement membranes, especially with glomerular structures (Cotran et al. 1999). Highly cationic molecules (as is the case for *Loxosceles* venom toxins) tend to bind to glomerular basement membrane anionic sites (proteoglycans) (Cotran et al. 1999). In addition, the glomerular barrier function is dependent on the molecular mass of proteins. Molecules with mass larger than 70 kD are less permeable than low molecular mass proteins (as is the case for a large number of *Loxosceles* venom toxins) (Cotran et al. 1999). This charge and size properties of venom toxins can account for their binding to glomerular basement membranes in a first step and later to tubule structures accumulated in tubule epithelial cells.

On the basis of the above findings, we have identified a possible cellular and molecular mechanism for the nephrotoxicity that occurs after envenomation by *Loxosceles* spiders. Although the renal injuries occurring after envenomation can be increased by dermonecrotic products and hemolysis, we conclude that *Loxosceles* venom toxins are direct and potentially nephrotoxic agents.

We hope that this report will bring some insight into loxoscelism, opening the possibility for a rational basis for therapy after brown spider bites.

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Experimental evidence of brown spider (*Loxosceles intermedia*) venom activity upon adhesive structures of human tumour cell lines

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Abstract

Brown spider venom has been reported to trigger platelet aggregation and act upon erythrocytes causing haemolysis. The venom has an inhibitory effect on neutrophil *in vitro* as well as cytotoxic effects to human umbilical vein and rabbit blood vessel endothelial cells. Additionally, the venom has deleterious effect upon mouse renal tissue structures and cells. Therefore, based on such cytotoxic activities, we postulated similar actions upon tumour cells. Human tumour cell lines Mel-85 and A-2058 (melanoma), MCF-7 and PMC-42 (breast adenocarcinoma), HeLa (uterine cervix adenocarcinoma), K-562 (leukaemia), DU-145 (prostate carcinoma) and A-172 (glioblastoma) were incubated with the venom and their viability was evaluated by MTT method. The cell viability evaluated over 24 hours in the presence of 100∝g/ml of the venom was 67% for Mel-85, 82% for A-2058, 52% for HeLa, 100% for MCF-7, 94% for PMC-42, 100% for K-562, 56% for DU-145, and 97% for A-172 cells. When venom at 200∝g/ml was used the cell viability was 48% for Mel-85, 68% for A-2058, 48% HeLa, 100% MCF-7, 86% for PMC-42, 100% for K-562, 45% for DU-145, and 100% for A-172. The venom treatment interferes in morphology of all cell lines studied, when changing their characteristic spread morphologies to a round shape. Cell biology studies using immunofluorescence techniques and confocal microscopy were carried out to evaluate morphological alterations triggered by the venom. Focal adhesion points and actin filament arrangement were disturbed as well as the binding of venom toxins on cell surface. In addition, venom treatment also hydrolysed the

extracellular matrix. In conclusion, the present results point to brown spider venom effect upon tumour cell morphology and open the possibility of using venom toxins as valuable tools for cell biology research.

keywords: tumour cell lines, adhesive structures, toxins, brown spider.

Introduction

The term Loxoscelism denotes a group of signs and symptoms triggered by the bite of brown spiders (*Loxosceles* genus spiders). Accidental bites caused by brown spiders have been reported to evoke two major clinical disturbances. The necrotic lesions evolve from oedema, erythema and local haemorrhage at the bite site to a characteristic lesion of skin with a gravitational spread (a hallmark of Loxoscelism). At the systemic level the envenomation may cause weakness, malaise, vomiting, fever and renal disturbances as well as haematological problems such as haemolytic anaemia, thrombocytopenia and disseminated intravascular coagulation (Futrell, 1992; Lung and Mallory, 2000; da Silva et al., 2004).

Loxosceles sp. venom is a complex mixture of proteic toxins especially enriched with molecules of low molecular mass in the range of 5-40 kDa (da Silveira et al., 2002; da Silva et al., 2004). Several toxins have been identified and well characterised biochemically; moreover their activities can explain venom cytotoxicity. These include sphingomielinases D of 32-35 kDa that have been shown to evoke necrotic lesions, haemolysis and platelet aggregation (Futrell, 1992; da Silva et al., 2004), hyaluronidase activities described as 33 and 63 kDa enzymes that could be related to cause the gravitational spreading of skin lesion (Young and Pincus, 2001; Veiga et al., 2001a). A metalloprotease of 32-35 kDa with gelatinolytic activity and another of 20-28 kDa with fibronectinolytic and fibrinogenolytic activities are both molecules that can play a role in the haemorrhage into the dermis, morphologic alterations of blood vessels and

defective wound healing as well as venom dissemination throughout the body (Feitosa et al., 1998; da Silveira et al., 2002). Serine-proteases at 85-95 kDa region (Veiga et al., 2000a) and lipase and alkaline phosphatase (Futrell, 1992, da Silva et al., 2004) has also been described. In addition, several other molecules and activities were identified in the venom, including proteolytic activities upon entactin, basement membranes and the protein core of heparan sulphate proteoglycan from endothelial cells, however, the venom toxins that are responsible for these activities remain to be determined (Veiga et al., 2001a).

Several direct and indirect cytotoxicity activities triggered by the brown spider venom have been described. The venom has a direct haemolytic effect on erythrocytes (Futrell, 1992; Williams et al., 1995; da Silva et al., 2004). Majestik et al. (1977) described an inhibitory venom effect on neutrophil chemotaxis *in vitro*. The venom has a direct activity on platelets evoking its aggregation (Futrell, 1992; Veiga et al. 2000b). Patel et al. (1994) demonstrated the venom cytotoxicity on vein endothelial cells (HUVEC). Additionally, Veiga et al. (2001b) described the deleterious effects of the venom upon rabbit blood vessel endothelial cells (CLPS). The venom acts on EHS (Engelbreth-Holm-Swarm) basement membrane integrity (Veiga et al., 2000b). Zanetti et al. (2002) and Ospedal et al. (2002) further demonstrated through histopathological findings that the venom has noxious effects upon skin blood vessel cells of rabbits. Luciano et al. (2004) showed direct cytotoxicity of venom in mouse kidney. The venom binds to glomerular and tubular basement membranes besides tubular epithelial cells.

Therefore, based on cytotoxic properties demonstrated by animal venom and plants, preliminary results describing toxin antitumoural effects (Richardson

and Ireland, 2004), we postulated some similar effects on tumour cell lines. In the present study we evaluated the effects of *L. intermedia* venom upon eight human tumour cell lines. Venom have altered viability, morphology and adhesive structures of assayed cells.

Materials and Methods

Materials

Monoclonal antibody that recognises the β_1 integrin subunit and polyclonal antibody for talin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Polyclonal antibodies to *L. intermedia* venom toxins were produced in a rabbit using crude venom as previously described (Luciano et al., 2004). Dulbecco's Modified Eagle's medium (DMEM), RPMI1640 medium and foetal calf serum (FCS) was obtained from Cultilab (Campinas, Brazil); penicillin and gentamycin were purchased from GIBCO (Bethesda, USA). Paraformaldehyde (20%, aqueous solution) was from Electron Microscopy Sciences (Washington, USA). DAPI (4´,6-diamino-2-phenylindole-HCl) and phalloidin labelled with FITC were purchased from Molecular Probes (Eugene, USA) and the aqueous mounting medium, Fluormount-G® from E.M.S. MTT and glycine were obtained from Sigma (St. Louis, USA). DMSO was purchased from Merck (Rio de Janeiro, Brazil). All other reagents were commercial products of the highest available purity grade.

Spider venom extraction

The venom was extracted from spiders captured from the wild and kept for a week without any food but with water *ad libitum.* The venom was extracted from the fangs of spiders by electrostimulation (15V) applied to the cephalothorax and was collected with a micropipette, dried under vacuum and frozen at –85°C until use. Pools of venom collected from 100 to 200 spiders in different batches were used during all experiments (Feitosa et al., 1998). Protein content was determined

by the Coomassie Blue method (Bradford, 1976). Venom was solubilised in phosphate buffered saline (PBS) at 2 mg/ml for its experimental use.

Cell lines and culture conditions

In this study, human cell lines used were: Mel-85 and A-2058 (human melanomas), HeLa (human cervix adenocarcinoma), MCF-7 and PMC-42 (human breast adenocarcinomas), K-562 (human chronic leukaemia), DU-145 (human prostate carcinoma) and A-172 (human glioblastoma), were kindly provided by Dr Ricardo R. Brentani from Ludwig Institute for Cancer Research (São Paulo, Brazil). Cell lines were chosen based on aggressive tumours (melanoma and glioblastoma), differing in histological origin (melanoma and glioblastoma lines have neuro-ectodermic origin, carcinoma lines have ectodermic origin and leukaemia line has mesodermic origin) and additionally epidemiology (breast and prostate). Cells were maintained in liquid nitrogen with a low number of passages. After thawing, cells were grown in monolayer cultures in the appropriate media containing gentamycin (50∝g/ml) and 10% foetal calf serum. Mel-85, HeLa, PMC-42 and K-562 cells were grown in RPMI 1640 medium and A-2058, DU-145, A-172 and MCF-7 were grown in DMEM medium. The cultures were kept at 37°C in a humidified atmosphere plus 5% CO_2. Release of Mel-85 and HeLa cells was performed by treatment for a few minutes with a 2mM solution of ethylenediaminetetraacetic acid (EDTA) in cation-free/PBS, all other adherent cells were harvested by using a treatment with 0,025% trypsin. The K-562 cell line is anchorage independent and grows in suspension. After counting, the cells were

then resuspended in an adequate volume of the respective medium supplemented with FCS and again plated in the presence or absence of brown spider venom.

Cell viability assay

Human tumour cells (6 x 10^3cells/well) were grown on 96-well plates (TPP) in RPMI or DMEM media, as previously described, containing 10% FCS for 16h. Then, medium was replaced by a serum-free one. After 24 hours, serum-free media were again replaced with media containing 10% FCS and the venom, at the concentrations of 50, 100 and 200∝g/ml in pentaplicates. Controls consisted of cultures in the absence of venom and presence of adequate amounts of PBS. After 24 hours, the viability was determined by using the MTT method (Freshney, 2000). The plates were read using a MicroELISA (Bio-Rad, Madison, USA) at 550nm for sample and 655nm for reference. Cell viability of control group (absence of venom) was normalised to 100%.

Statistical analysis

Statistical analysis of viability data was performed using analysis of variance (ANOVA) and the Tukey test for average comparisons. Mean ± S.D. values were used. Significance was determined as $p < 0.05$.

Immunofluorescence and Fluorescence Cytochemistry

MCF-7 cells were replated on glass coverslips (13mm diameter) for seven days then cells were incubated with 100∝g/ml of brown spider venom for 24 hours

at 37°C under a humidified atmosphere plus 5% CO_2. In the control group, the appropriate medium contained adequate amounts of vehicle PBS. Cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 30 minutes at 4°C. Cells on coverslips were incubated with 0.1M glycine for 3 minutes, washed with PBS and then blocked with PBS containing 1% BSA for 30 minutes at room temperature (25°C). Integrin β_1 subunit, talin and venom toxins were detected with specific antibodies as described in the Materials section. Briefly, cells were incubated with primary antibodies (for integrin β_1 subunit and talin diluted 0.6∝g/ml and for venom toxins diluted 1.0∝g/ml in PBS-1% BSA) for 1 hour at room temperature. After washing three times with PBS the cells were subsequently incubated with secondary antibodies conjugated with rhodamine for β_1 integrin and talin detections at room temperature for 40 minutes. For venom toxins, the secondary antibody used was fluorescein isothiocyanate-conjugate and experiments were carried out under the same experimental conditions. In order to decorate actin fibers, cells were treated with fluorescein-phalloidin (1:200 in PBS) for 40 minutes. After washing, samples were mounted with Fluormont-G® and observed under a fluorescence confocal microscope (Confocal Radiance 2,100, BioRad, Hercules, USA) coupled to a Nikon-Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division, Melville, USA). For nuclear fluorescence cytochemistry, cells were incubated with DAPI (0.5∝g/ml diluted in PBS) for 5 minutes. The samples were washed and observed under a confocal microscope as mentioned above.

Periodic acid-Schiff Method

MCF-7 cells replated on glass coverslips for seven days were fixed in modified Carnoy's fixative for 3 hours (5% acetic acid instead of 10% as originally proposed). After fixation, materials were incubated with the venom (100∝g/ml) at room temperature and processed for detection of glycoconjugates by the periodic acid-Schiff method (Culling et al., 1985). The samples were observed under a light microscope (Zeiss Axiophot, Göttingen, Germany).

Results

Effects of *L. intermedia* venom on the cytotoxicity of different tumour cell lines

The venom cytotoxicity evaluation was carried out in the presence of 50, 100 and 200∝g/ml of venom for 24 hours. There were different responses, according to the concentration and cell lines tested; these results are summarised on Table 1. After 24 hours, the lowest venom concentration (50∝g/ml) had little effect on the majority of cell lines studied. Under this condition, viability after venom exposure was around 84% for Mel-85, 94% for A-2058, 62% for HeLa, 98% for MCF-7, 99% for PMC-42, 100% for K-562, 76% for DU-145 and 100% for A-172 cells when compared to cells in the absence of venom (control 100%). Venom exposition to 100∝g/ml reduced viability to 67% for Mel-85, 82% for A-2058, 52% for HeLa, 100% for MCF-7, 94% for PMC-42, 100% for K-562, 56% for DU-145 and 97% for A-172. At 200∝g/ml, the viability was 48% for Mel-85, 68% for A-2058, 48% for HeLa, 100% for MCF-7, 86% for PMC-42, 100% for K-562, 45% for DU-145 and 100% for A-172 when compared to control cells (100%).

L. intermedia venom activity on the morphology of different tumour cell lines

Furthermore, the activity of the venom on the morphology and adherence to culture substratum of tumour cell lines were analysed. K-562 cell line was not considered in this evaluation since it is anchorage independent and grows in suspension. The obtained results (Figure 1) showed very similar phenomena for all

cell lines studied. Mel-85 and A-2058 melanoma cells even after 72 hours exposed to the venom presented little alterations on morphology even when treated with 200 ∝g/ml. All other assayed lines after 72 hours of incubation the number of cells with round shape enhanced dramatically and cells detached from the culture substratum. But even in supernatant they presented a round shape and appeared as aggregated groups. These cell-venom culture treatments demonstrated that the venom had an effective activity on assayed cell line morphology.

Effects of *L. intermedia* venom upon β_1 integrin distribution, focal adhesion contacts and cytoskeletal rearrangements

With the objective to explain how venom acts on cell morphology, MCF-7 cells (that practically suffered no venom actions on viability and proliferation, but after exposure to the venom it showed changes on morphology becoming rounded) were incubated with 100∝g/ml of venom at similar conditions as described above. Immunocytolabelling for β_1 integrin (receptors for extracellular matrix constituents) and focal contacts (which are highly specialised points of attachment of cells mediated by integrin at cell surface and actin filaments inside the cell, linked by accessory proteins as vinculin and talin), were carried out. In addition, actin filaments were labelled with fluorescent phalloidin. As described in Figure 2, it was observed that venom treatment caused an apparent rearrangement of assembled stress fibers of actin (control) to the entire circumference of the cell or patches of actin distributed throughout the cell cytoplasm although in some cases these patches were clustered. The β_1 integrin profile of control cells showed a typical

linearly punctuate staining at cell-cell borders or cell surface of adhered cells, and after venom treatment this organisation suffered a change to a linear pattern of localisation at the edge cells. Similarly, organised focal contacts distributed on the surface of adhered cells (control) changed to patches localised on the periphery of venom treated cells.

Extracellular matrix (ECM) disorganisation and toxins-cell binding after *L. intermedia* venom exposure

Finally, with the intent to demonstrate that venom toxins interact with cellular structures or extracellular molecules, MCF-7 cells treated with the venom were studied. Cells were analysed for binding venom toxins, β_1 integrins, cell surface profile, nuclei and ECM distribution and organisation. As shown on Figure 3, venom toxins bind to cell indicating an immunofluorescent pattern of deposition as clustered structures on cell surface. There was a coalignment of venom deposition on cell surface together with integrins (a cell surface marker), but they showed different distributions since integrins appear linearly distributed and venom toxins as clustered forms. Details of double staining through immunofluorescence procedures for β_1 integrins and venom toxins supported some coalignment for both molecules bound to cell surface. On the other hand (Figure 4), cells treated with the venom had their pattern of staining for ECM decreased when compared to control cells supporting the idea that the venom effectively disrupted ECM.

Discussion

The biological activities of animal venom on living organisms and cells have been the subject of analysis in laboratories throughout the world. Investigations in such areas have yielded a great deal of important information on biological systems. In fact, nowadays venom toxins have been indicated as promising powerful reagents and tools for diseases treatment and as keys to the design of new medicine.(Soutar and Ginsberg, 1993; Markland, 1998). In addition, venom toxins and cell culture protocols can also be excellent tools for investigating molecular and cellular mechanisms. In this area, for instance, *Loxosceles spp* venom toxins have been attracting great biotechnological attention. Studies with *L. reclusa* venom described a product named ARACHnase, which is plasma that mimics the presence of a lupus anticoagulant and provides a positive control for lupus anticoagulant testing (McGlasson et al., 1993). Moreover, research in the area of *Loxosceles sp.* venom has been producing monoclonal antibodies to venom toxins that can be used as reagents for clinical applications, for immunoaffinity purification or screening of venom toxins (Guilherme et al., 2001, Alvarenga et al., 2003, da Silva et al., 2004). Likewise, production of recombinant *Loxosceles* venom proteins has been generating enough reagents that will improve the knowledge in the area, as well as for medical applications (Kalapothakis et al., 2002, Pedrosa et al., 2002, Araujo et al., 2003).

The aim of this work was to describe and evaluate possible cytotoxic activities of *L. intermedia* venom on different human tumour cell lines, as well as to shed some insights upon the comprehension of how venom toxins trigger these

noxious effects upon cells. Indeed, literature data have described several examples of cytotoxic activities of *Loxosceles* venom on different cell models (see Introduction). However, little is known about venom effects upon tumour cell lines, or its potential effect on tumour models. Herein, by using 50∝g (this concentration represents a value near the average concentration of venom proteins injected during accidents), 100∝g and 200∝g of venom, we reported evidence that *L. intermedia* venom has cytotoxic activities on adhesive structures of human tumour cell lines of different histological sources and especially on disturbances of cell morphology. Experiments to evaluate cytotoxicity suggested that the venom triggers different responses in the inhibition of tumour cell line viability. Venom cytotoxic effect was most active on HeLa cells, with an inhibition in the order of 50%. Inhibition upon other cells was consistent with the venom doses used and changed according to individual characteristics of cells. Apparently, at the experimental conditions assayed, the melanoma cell line A-2058, adenocarcinoma cell lines MCF-7 and PMC-42 together with the glioblastoma cell line A-172 and chronic myelogenous leukaemia cell line K-562 were more resistant to the venom actions compared to more susceptible cell lines as HeLa (cervix tumour cell line) and DU-145 (prostate tumour). Such sensitivity differences could be putatively explained by multidrug resistance apparatus normally found in aggressive tumours (as is the case of melanoma, glioblastoma and some leukaemia tumour cells), which pump toxic molecules outside cells increasing their resistance (Alberts et al., 2002). In addition, differences in tumour cell lines ECM could also contribute to this individual characteristics, since the ECM regulates cellular growth, adhesion,

migration and differentiation events which are very important in tumour biology (Mendelsohn et al., 2001; Alberts et al., 2002).

Clustered immunofluorescent pattern of venom toxins bound to the cell surface suggested venom deposition along specific regions of the cytoplasmic cell membrane or cell surface, rather than an unspecific venom adsorption on the cell membrane that would induce a linear and diffused immunofluorescent pattern.

The venom altered the morphology of all cell lines changing their aspect to a round shape. Its putative activity was postulated either on the extracellular matrix organisation or on cellular structures involved in adhesiveness and spreading and for guiding cell migration as cell surface receptors and cytoskeleton. It has also shown that venom treatment deeply altered the actin filament arrangements. Actin filament bundles (in control cells) suffered disorganisation in length and localisation after cell exposure to the venom. Likewise, focal adhesive points were reorganised at the cell periphery of the round cells.

The extracellular matrix molecules can alter the dynamic behaviour of cytoskeleton through transmembrane adhesion proteins especially integrins (Calderwood et al., 2000 and Alberts et al., 2002). The coalignment of venom toxins on cell surface (as evidenced by confocal microscopy) and the decreasing of ECM staining strongly supported that venom exposure disorganised or degraded ECM molecules, which could trigger disorganisation of β_1 integrin receptors, focal contact points and cytoskeleton. Since previous data have described *L. intermedia* venom action upon extracellular matrix constituents such as fibronectin, entactin, fibrinogen and heparan sulphate proteoglycan (Feitosa et al., 1998; Veiga et al.,

1999; Veiga et al., 2000b; Veiga et al., 2001a; 2001b; Zanetti et al., 2002), it was postulated that the defective adhesion and morphology of venom treated cells can be explained by some degradation and disorganisation of extracellular matrix molecules and consequent rearrangements of focal adhesion points and/or cytoskeleton. However, a direct venom activity on β_1 integrins can not be ruled out. In regard to this, but contrary to what could be expected by venom treatment of cells, it apparently altered β_1 integrin cell surface profile but did not decrease the positivity of these molecules, suggesting that the venom did not cause β_1 integrin hydrolysis. Identically, a venom-direct effect on cytoskeleton and especially upon actin filaments or the various actin-associated proteins can not be discarded since cytoskeletal rearrangements are also mediated by several accessory proteins (Sastry and Burridge, 2000; Alberts et al., 2002).

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Legends

Figure 1. Effects of *L. intermedia* venom on the morphology of different human tumour cell lines.

Seven different human tumour cell lines exposed to the venom were observed in an inverted microscope. The morphological alterations of cells included changing of their characteristic morphology to a round shape, loss of adhesion and appearance of aggregated cells in suspension according to individual susceptibility to the venom. Control cells are shown on the left (magnification X200).

Figure 2. Effects of *L.intermedia* venom on the organisation of adhesive structures of MCF-7 tumour cells.

Lanes A, C and E represent control cells and lanes B, D and F venom-treated cells. Lanes A and B represent cells incubated with primary antibodies to β_1 integrin subunit (arrows point for β_1 integrin cell localisation and arrow-heads show nuclei). Lanes C and D depict cells incubated with primary antibodies to talin (arrows represent focal adhesion points distribution and arrow-heads nuclei).

Lanes E and F show cells incubated with phalloidin-fluorescein conjugate (arrows depict for actin organisation and arrow-heads show nuclei) (magnification x2400).

Figure 3. *L.intermedia* venom toxins binding to MCF-7 cell surface

Lane A depicts control for specificity of toxin antibodies. Cells at absence of venom incubated with antibodies for venom toxins and specific secondary fluorescent conjugate (arrow-heads point for nuclei stained by DAPI). Lane B shows control for specificity of secondary-conjugate. Cells exposed to the venom incubated only with fluorescein conjugate-secondary antibodies (arrow-heads show nuclei staining). Lanes C and D depicted venom-treated cells immunostained for β_1 integrin localisation (arrows) and venom toxins deposition (arrows) respectively, on MCF-7 cell surface (arrow-heads show nuclei staining). Lane E represents double staining for β_1 integrin (closed arrows) and venom toxins binding on MCF-7 cell surface (open arrows) (magnification x2400). Lane F depicts for details of venom toxins to MCF-7 cells. Venom toxins seem heterogeneously distributed along the cell as cluster of patches of different sizes with some of them being shed (open arrows). Closed arrow shows β_1 integrin localisation on cell surface (magnification x4800).

Figure 4. Effects of L. intermedia venom on the organisation of extracellular matrix produced by MCF-7 cells

For visualisation of the extracellular matrix (ECM), samples were stained with periodic acid-Schiff reagent (PAS) and analysed through light microscope. Photomicrographs depict (A) MCF-7/ECM normal organisation and distribution

(control) and (B) MCF-7/ECM exposed to the venom. Arrows point for ECM constituents and organisation (magnification x400).

Table 1: Effects of *L. intermedia* venom on the viability of different human tumour cell lines.

The venom cytotoxicity was measured after 24h at indicated concentrations. Experiments were performed in pentaplicates and values given are the mean ±S.D. Significance is defined as $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

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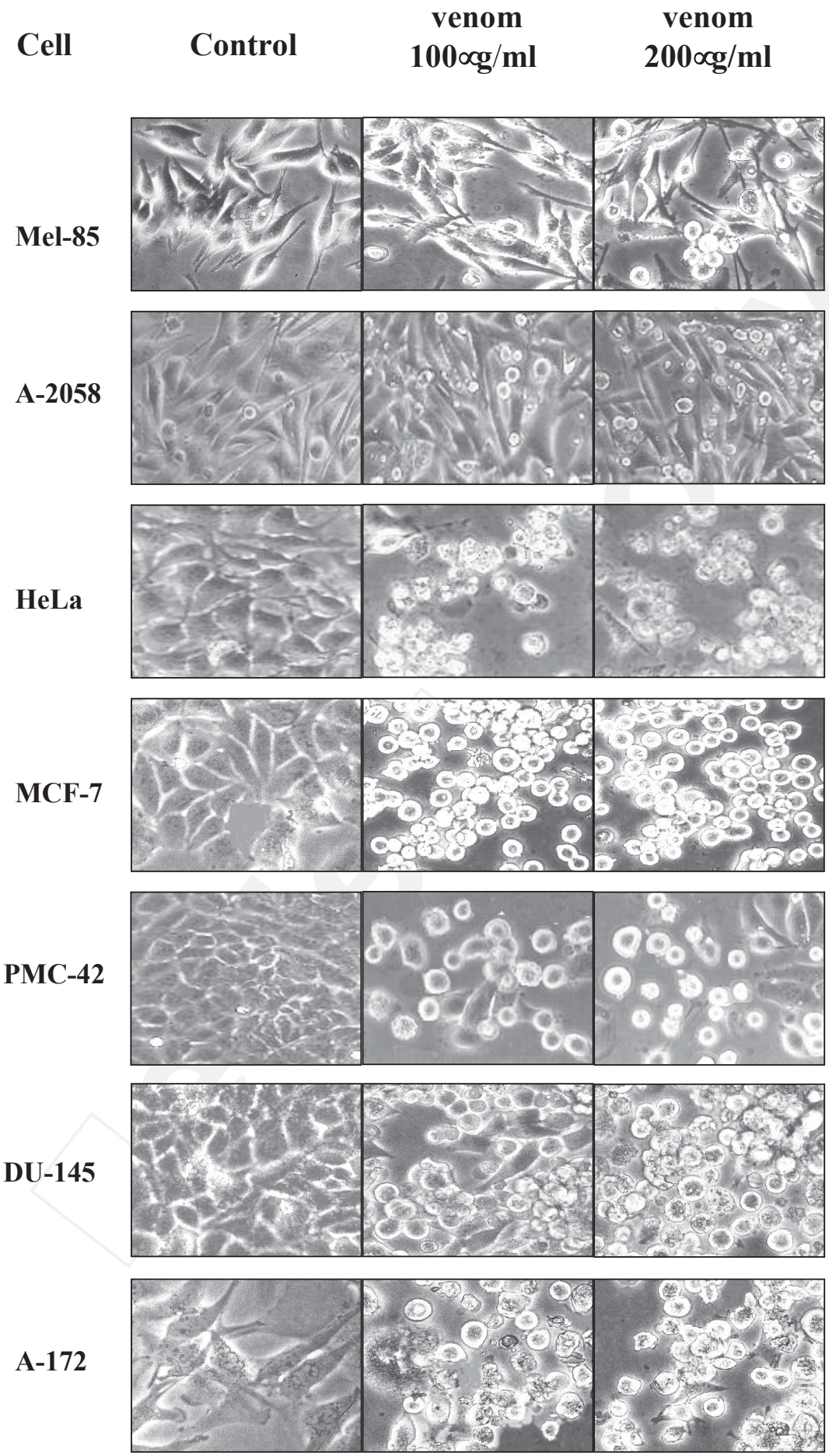
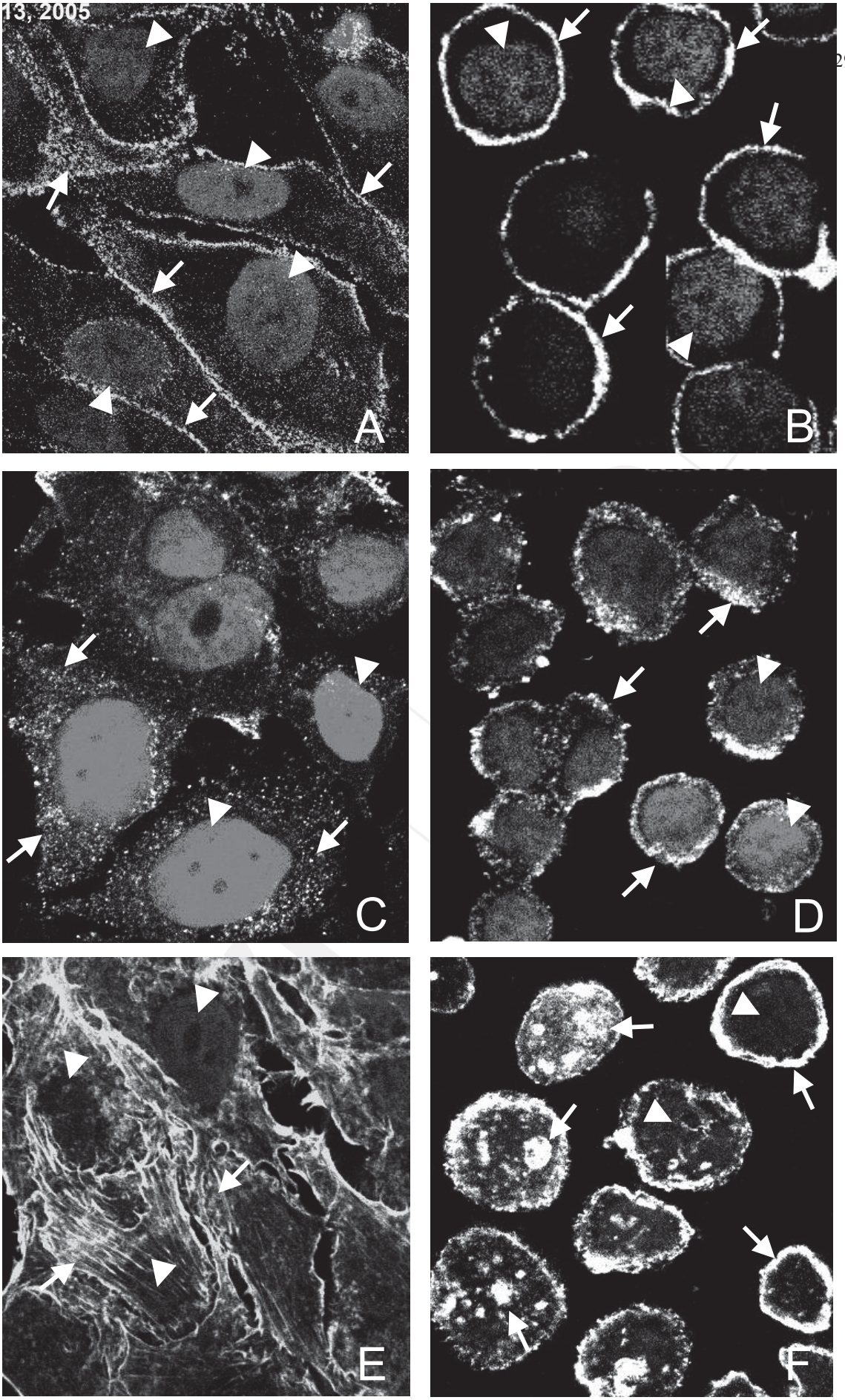


FIGURE 1: Creplive et al., 2005



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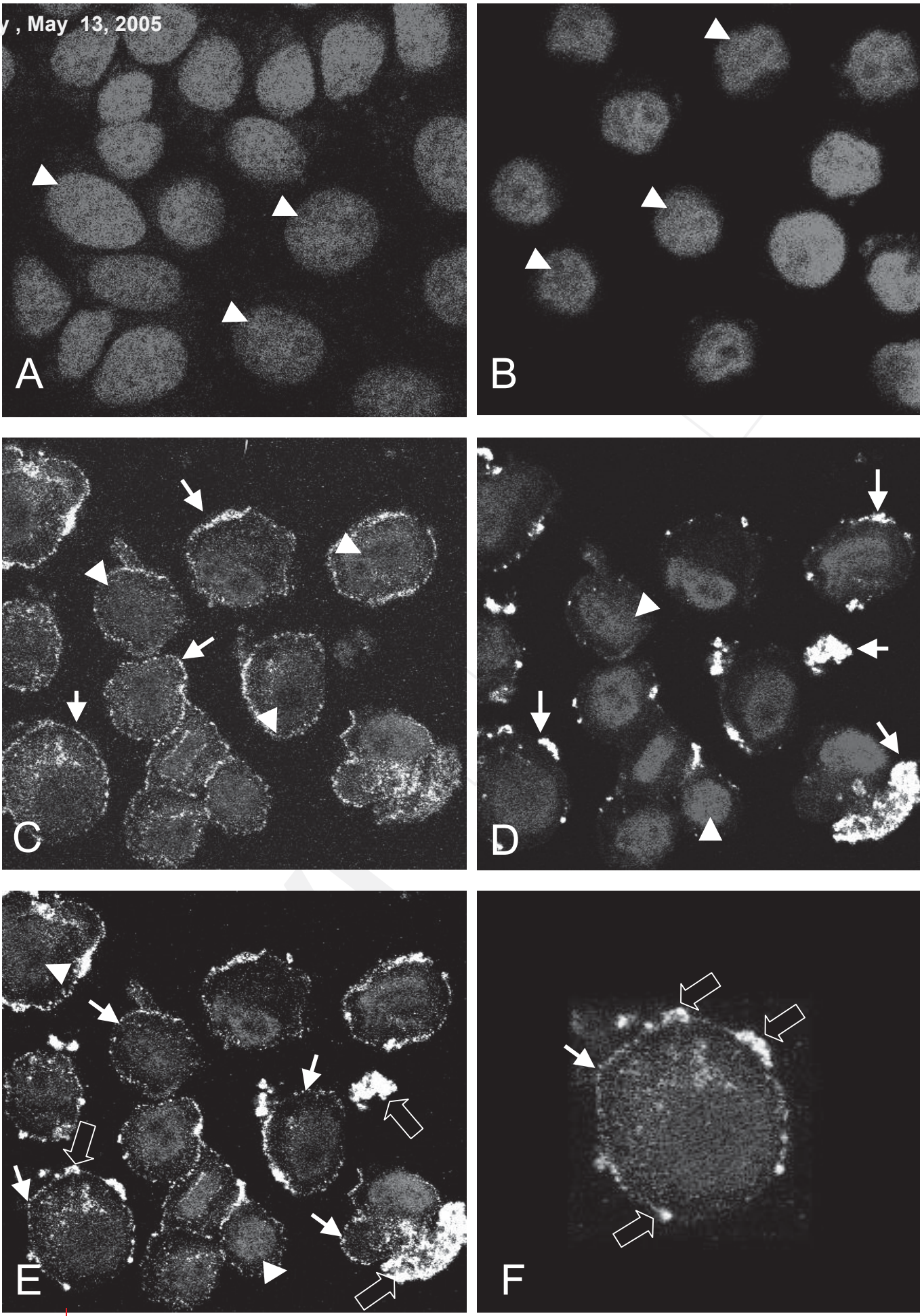

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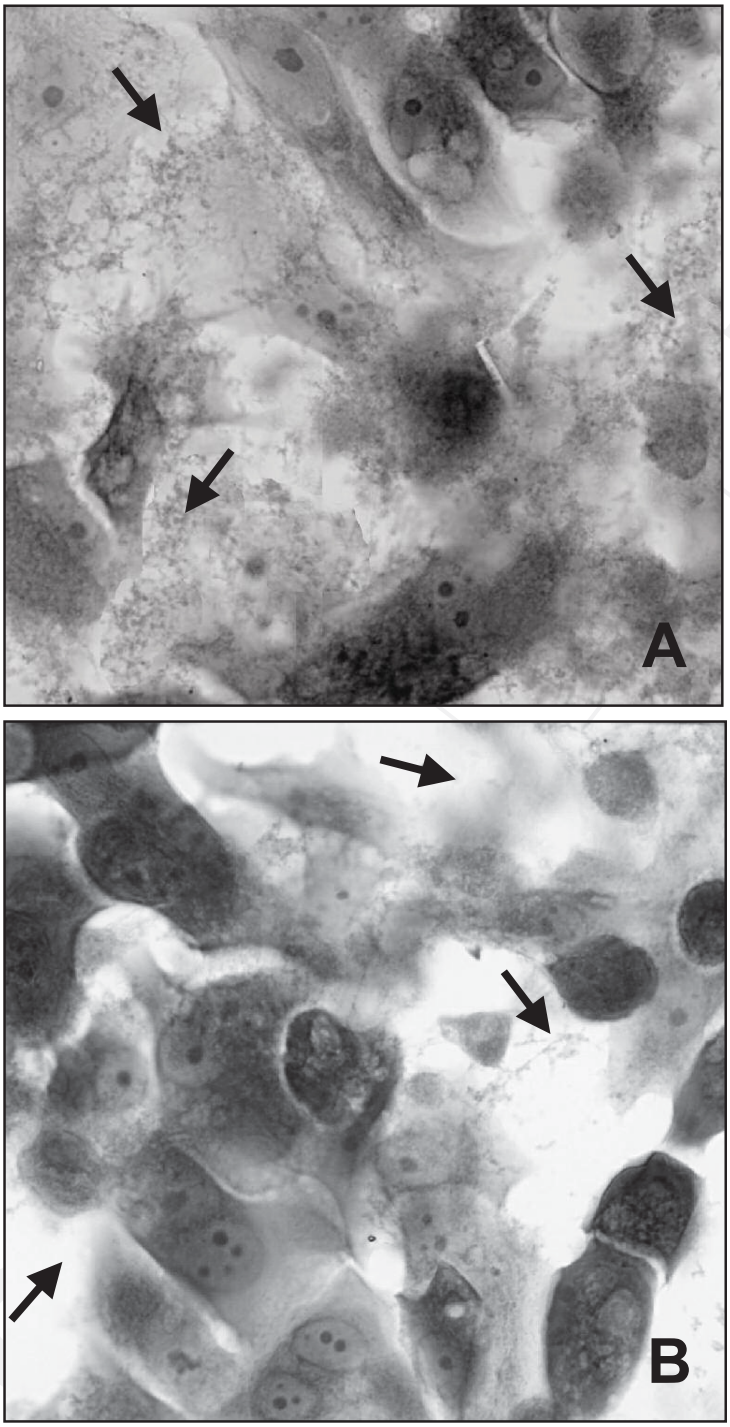

FIGURE 4: Creplive *et al.*, 2005

Table 1: Effects of the *Loxosceles intermedia* venom on the viability of different human tumour cell lines.

Cell lines	Venom 50∝g/ml	Venom 100∝g/ml	Venom 200∝g/ml
Mel-85	84%±20	67%±8,0**	48%±11***
A-2058	94%±4,0	82%±3,0***	68%±4,0***
HeLa	62%±10**	52%±8,0***	48%±7,0***
MCF-7	98%±11	100%±5,0	100%±10
PMC-42	99%±3,0	94%±5,0	86%±6,0***
K-562	100%±12	100%±8	100%±15
DU-145	76%±5,0***	56%±2,0***	45%±6,0***
A-172	100%±10	97%±7	100%±7

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Brown spider dermonecrotic toxin directly induces nephrotoxicity

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Abstract

Brown spider (*Loxosceles* genus) venom can induce dermonecrotic lesions at the bite site and systemic manifestations including fever, vomiting, convulsions, disseminated intravascular coagulation, hemolytic anemia and acute renal failure. The venom is composed of a mixture of proteins with several molecules biochemically and biologically well characterized. The mechanism by which the venom induces renal damage is unknown. By using mice exposed to *Loxosceles intermedia* recombinant dermonecrotic toxin (*LiRecDT*), we showed direct induction of renal injuries. Microscopic analysis of renal biopsies from dermonecrotic toxin-treated mice showed histological alterations including glomerular edema and tubular necrosis. Hyalinization of tubules with deposition of proteinaceous material in the tubule lumen, tubule epithelial cell vacuoles, tubular edema and epithelial cell lysis was also observed. Leukocytic infiltration was neither observed in the glomerulus nor the tubules. Renal vessels showed no sign of inflammatory response. Additionally, biochemical analyses showed such toxin-induced changes in renal function as urine alkalinization, hematuria and azotemia with elevation of blood urea nitrogen levels. Immunofluorescence with dermonecrotic toxin antibodies and confocal microscopy analysis showed deposition and direct binding of this toxin to renal intrinsic structures. By immunoblotting with a hyperimmune dermonecrotic toxin antiserum on renal lysates from toxin-treated mice, we detected a positive signal at the region of 33–35 kDa, which strengthens the idea that renal failure is directly induced by dermonecrotic toxin. Immunofluorescence reaction with dermonecrotic toxin antibodies revealed deposition and binding of this toxin directly in MDCK epithelial cells in culture. Similarly, dermonecrotic toxin treatment caused morphological alterations of MDCK cells including cytoplasmic vacuoles, blebs, evoked impaired spreading and detached cells from each other and from culture substratum. In addition, dermonecrotic toxin treatment of MDCK cells changed their viability evaluated by XTT and Neutral-Red Uptake methodologies. The present results point to brown spider dermonecrotic toxin cytotoxicity upon renal structures in vivo and renal cells in vitro and provide experimental evidence that this brown spider toxin is directly involved in nephrotoxicity evoked during *Loxosceles* spider venom accidents.

Keywords: Brown spider; Dermonecrotic toxin; Cytotoxicity; Kidney; MDCK cells

Introduction

Accidents involving spiders of the genus *Loxosceles* (brown spiders) have been reported in North America, Latin America, Europe, Middle East and other parts of Asia, Africa and Australia (Futrell, 1992; da Silva et al., 2004). In the USA, the range of *Loxosceles* spiders extend from southeastern Nebraska to southernmost Ohio and south into Georgia and most of Texas. Brown spiders inhabit also Arizona, Nevada, New Mexico, Utah and southern California (Futrell, 1992; da Silva et al., 2004;

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Vetter and Bush, 2002). Envenomation caused by brown spider gives rise to dermonecrotic lesions with gravitational spreading (the hallmark of bites) and systemic manifestations such as renal failure, disseminated intravascular coagulation and intravascular hemolysis (Futrell, 1992; da Silva et al., 2004). Systemic involvement is less common than skin injuries, but it may also be the cause of complications and death.

The mechanisms by which *Loxosceles* spider venom causes its lesions are currently under investigation. The venom is a mixture of proteic toxins enriched with molecules of low molecular mass (5–40 kDa) (Mota and Barbaro, 1995; da Silva et al., 2004; da Silveira et al., 2002). Several toxins have been identified and well characterized biochemically in *Loxosceles* venom. These include alkaline phosphatase, ribonucleotide phosphohydrolase, hyaluronidase, serine proteases, metalloproteases and sphingomyelinase-D (Feitosa et al., 1998; Futrell, 1992; da Silva et al., 2004; Veiga et al., 2001a). Metalloproteases named Loxolysin A (20–28 kDa) and Loxolysin B (32–35 kDa) have gelatinolytic, fibronectinolytic and fibrinogenolytic activities and can play a role in hemostatic disturbances occurring after envenomation such as injury of blood vessels, hemorrhage into the dermis, imperfect platelet adhesion and defective wound healing (Feitosa et al., 1998; da Silveira et al., 2002; Zanetti et al., 2002). The hyaluronidase toxin degrades hyaluronic acid and chondroitin sulfate residues from proteoglycans and could be putatively involved in the gravitational spreading of dermonecrosis and as systemic spreading factor (Futrell, 1992; da Silva et al., 2004; Young and Pincus, 2001). The sphingomyelinase-D (30–35 kDa), also called dermonecrotic toxin, is the best biochemically characterized molecule identified in the venom of different *Loxosceles* species. This toxin, as a native molecule or as recombinant variants, can induce dermonecrosis, platelet aggregation and experimental hemolysis (Cunha et al., 2003; Kalapothakis et al., 2002; Pedrosa et al., 2002). Recently, de Castro et al. (2004) identified a family of low molecular mass (5.6–7.9 kDa) insecticidal toxins in the *Loxosceles intermedia* venom. The authors postulated that these molecules might contribute to the toxicity of the venom. Other activities produced by unidentified toxins have been described in the venom. These include hydrolytic activities in the protein core of a heparan sulfate proteoglycan from vessel endothelial cells, entactin and basement membranes (Veiga et al., 2000, 2001a, 2001b). The mechanisms by which these activities play a role in the noxious effects of the venom have not been fully determined.

Brown spider venom contributes directly or indirectly to cytotoxic activities upon different cells. The venom has hemolytic activity on erythrocytes (Futrell, 1992; Williams et al., 1995) and causes platelet aggregation (Futrell, 1992; Veiga et al., 2000). The venom has a direct inhibitory effect on neutrophil chemotaxis in vitro (Majestik et al., 1977). On the other hand, it can induce a strong indirect dysregulated endothelial-cell-dependent neutrophil activation (Patel et al., 1994), which seems to play a role in dermonecrotic injuries evoked after envenomation (Futrell, 1992; da Silva et al., 2004). This last hypothesis is strengthened by histopathological findings from rabbits that were experimentally exposed to the venom (Elston et al., 2000; Ospedal et al., 2002) and from histological analysis of human patients after brown spider bites (Futrell, 1992; da Silva et al., 2004; Yiannias and Winkelmann, 1992). Additionally, the venom has a cytotoxic effect upon cultured human umbilical vein endothelial cells (Patel et al., 1994) and rabbit aorta endothelial cells (Veiga et al., 2001a).

Renal disorders evoked by brown spider venom have been earlier reported from clinical data of victims (Futrell, 1992; Lung and Mallory, 2000; da Silva et al., 2004). Venom nephrotoxicity was further demonstrated by histopathological findings from crude-venom-treated mice. Histological analysis of kidneys showed hyalinization and erythrocytes in the Bowman's space, glomerular collapse, tubular epithelial cell cytotoxicity and deposition of proteinaceous material within the tubular lumen. Immunofluorescence demonstrated the deposition and binding of venom toxin(s) along the tubular and glomerular structures (Luciano et al., 2004). We report here the direct effect of a recombinant dermonecrotic toxin obtained from a cDNA library of *L. intermedia* gland venom. The recombinant molecule was able to reproduce the nephrotoxicity evoked by crude venom. This toxin may account for renal injury associated with envenomation by *Loxosceles* spiders.

Methods

Reagents. Polyclonal antibodies to *L. intermedia* dermonecrotic toxin (*LiRecDT*) were produced in the rabbit as described by Luciano et al. (2004). Hyperimmune IgGs were purified from serum using Protein–A Sepharose (Amersham Biosciences, Piscataway, USA) as recommended by the manufacturer. Fluorescein-conjugated anti-rabbit IgG was purchased from Sigma, St. Louis, USA. Crude venom from *L. intermedia* was extracted from spiders captured in the wild as described by Feitosa et al. (1998).

cDNA library construction. Two hundred adult *L. intermedia* spiders were submitted to venom extraction by electrostimulation (15 V applied to the cephalothorax) to stimulate mRNA production. After 5 days, venom glands were collected, and mRNA was purified using the FastTrack 2.0 mRNA Isolation Kit, according to the manufacturer's protocol (Invitrogen, Carlsbad, USA). The cDNAs were synthesized from 4.3 μg mRNA using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning, linked to *Sal*I adaptors, *Not*I digested and linked to pre-cut *Not*I–*Sal*I pSPORT1 vector, using the

method recommended by the manufacturer (Invitrogen). *Escherichia coli* DH5α cells were transformed with ligation reaction and then plated on LB agar plates containing 100 μg/ml ampicillin.

cDNA library screening. Randomly chosen colonies were inoculated in 5 ml LB broth containing 100 μg/ml ampicillin, grown overnight at 37 °C (with aeration), and the DNA plasmid was purified by alkaline lysis method using QIAprep Spin Miniprep Kit following the manufacturer's protocol (QIAGEN, Valencia, USA). Purified plasmids were sequenced on both strands using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). Reactions were carried out on an ABI 377 automatic sequencer (Applied Biosystems), and the primers used to sequence were T7 promoter and SP6 promoter. The nucleotide sequences were analyzed using Genetyx-Mac v7.3 software (Software Development, Tokyo, Japan). The putative protein products from cDNA sequences were compared to GenBank protein databases at NCBI (Altschul et al., 1997).

Recombinant protein expression. The cDNA corresponding to the putative mature dermonecrotic protein was amplified by PCR. The forward primer used was 30 Rec sense (5′-CTCGAGGCAGGTAATCGTCGGCCTATA-3′) designed to contain an *Xho*I restriction site (underlined) plus the sequence related to the first seven amino acids of mature protein. The reverse primer used was 30 Rec antisense (5′-CGGGATCC**TTA**TTTCTTGAATGTCACCCA-3′), which contains a *Bam*HI restriction site (underlined) and the stop codon (bold). The PCR product was cloned into pGEM-T vector (Promega, Madison, USA). The pGEM-T vector containing the mature protein encoding cDNA was then digested with *Xho*I and *Bam*HI restriction enzymes. The excised insert was gel purified using QIAquick Gel Extraction Kit (QIAGEN) and subcloned into pET-14b (Novagen, Madison, USA) digested with the same enzymes. The correct construct was confirmed by sequencing. The recombinant construct was expressed as fusion protein, with a 6× His-tag at the N-terminus and a 13 amino acid linker including a thrombin site between the 6× His-tag and the mature protein (N-terminal amino acid sequence before the mature protein: MGSSHHHHHH-SSGLVPRGSHMLE). pET-14b/*L. intermedia* cDNA construct was transformed into One Shot *E. coli* BL21(DE3) pLysS competent cells (Invitrogen) and plated on LB agar plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. A single colony was inoculated into 50 ml LB broth (100 μg/ml ampicillin and 34 μg/ml chloramphenicol) and grown overnight at 37 °C. A 10 ml portion of this overnight culture was grown in 1 L LB broth/ampicillin/chloramphenicol at 37 °C until the OD at 550 nm reached 0.5. IPTG (isopropyl β-D-thiogalactoside) was added to a final concentration of 0.05 mM, and the culture was induced by the incubation for additional 3.5 h at 30 °C (with vigorous shaking). Cells were harvested by centrifugation (4000 × *g*, 7 min), and the pellet was frozen at − 20 °C overnight.

Protein purification. Cells were disrupted by thawing, and the harvested cell was pasted in 40 ml of extraction buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme). Lysed material was centrifuged (20,000 × *g*, 20 min), and the supernatant was incubated with 2 ml Ni-NTA agarose beads for 1 h at 4 °C (with gentle agitation). The suspension was loaded to a column, and the packed gel was exhaustively washed with the appropriate buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole) until the OD at 280 nm reached 0.01. The recombinant protein was eluted with 20 ml of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 250 mM imidazole), and 1 ml fractions were collected and analyzed by 15% SDS-PAGE under reducing conditions. Fractions were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 8.0, containing 200 mM NaCl.

Animals. Adult Swiss mice weighing approximately 25–30 g and adult rabbits weighing approximately 4 kg from the Central Animal House of the Federal University of Paraná were used for in vivo experiments with crude venom and *LiRecDT*. All experimental protocols using animals were performed according to the "Principles of Laboratory Animal Care" (NIH Publication no. 85-23, revised 1985) and "Brazilian Federal Laws".

LiRecDT administration. For the evaluation of the dermonecrotic effect, 10 μg of *LiRecDT* diluted in PBS was injected intradermally into a shaved area of rabbit skin. Dermonecrosis was checked 24 h after injection as previously described by Veiga et al. (2000). Purified *LiRecDT* (samples of 1 mg of proteins/kg of mice) were diluted in PBS (150 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.3). These samples were injected intraperitoneally (to quicken and to make uniform the release of toxin into circulation) in a volume of 100 μl in each mouse. Intradermal injections induced similar animal manifestations, however, the time for this observation was greater and more variable (between 24 and 72 h, data not shown). The animals were divided into two groups, a control group and a test group. The control group consisted of five animals receiving only PBS, and the test group consisted of five animals receiving *LiRecDT*. During the experimental procedures, the envenomation was repeated 3 times, completing a number of 15 animals in the control group and 15 animals receiving *LiRecDT*. All animals were kept under the same experimental conditions.

Blood and urine collections and laboratory analyses. Blood samples (directly from the heart) were obtained from mice anesthetized with ketamine (Agribands, Paulínia, Brazil) and acepromazine (Univet, São Paulo, Brazil). Urine samples were obtained from mice submitted to soft massage

on the abdominal region and collected using a micropipette. Blood urea nitrogen and urinalysis were determined using standardized techniques and reagents as described by Henry (2001).

Gel electrophoresis and immunoblotting. Lysed renal cells were obtained from treatment of kidneys with a lysis buffer (50 mM Tris–HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 1 mM $CaCl_2$, 1 mM phenylmethanesulfonyl fluoride, 5 mM EDTA and 2 μg/ml aprotinin) for 15 min at 4 °C. The extract was clarified by centrifugation for 10 min at 13,000 × *g*. Protein content was determined by the Coomassie blue method (BioRad, Hercules, USA) as described by Bradford (1976). Renal extracts (100 μg of proteins) or purified *LiRecDT* (2 μg) were submitted to 10% SDS-PAGE under non-reducing conditions. For protein detection, gels were stained with Coomassie blue. For immunoblotting, proteins were transferred to nitrocellulose filters overnight as described by Towbin et al. (1979) and immunostained by using hyperimmune purified IgG which reacts to dermonecrotic toxin as described in Reagents. The molecular mass markers used were acquired from Sigma.

Histological methods for light microscopy. Rabbit skin and kidneys (mouse) were collected from animals anesthetized with ketamine (Agribands) and acepromazine (Univet) and then fixed in "ALFAC" fixative solution (ethanol absolute 85%, formaldehyde 10% and glacial acetic acid 5%) for 16 h at room temperature. After fixation, samples were dehydrated in a graded series of ethanol before paraffin embedding (for 2 h at 58 °C) (Drury and Wallington, 1980). Then, thin sections (4 μm) were processed for histology. Tissue sections were stained by hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) (Beautler et al., 1995; Culling et al., 1985).

Kidney sections and MDCK cells immunofluorescence assays. For immunofluorescence microscopy, kidney tissues were fixed with 2% paraformaldehyde in PBS for 30 min at 4 °C, incubated with 0.1 M glycine for 3 min and blocked with PBS containing 1% BSA for 1 h at room temperature. Histological sections were incubated for 1 h with specific antibodies raised against dermonecrotic toxin (2 μg/ml) as described in Reagents. The sections were washed three times with PBS, blocked with PBS containing 1% BSA for 30 min at room temperature and incubated with fluorescein-conjugated anti-rabbit IgG secondary antibodies (Sigma) at room temperature for 40 min. For antigen competition assay, the immunofluorescence protocol was the same as described above, except that the hyperimmune IgG to dermonecrotic toxin was incubated previously for 1 h with 10 μg/ml of *LiRecDT* diluted in PBS. Then, the mixture was incubated with renal sections identically as above. Alternatively, MDCK cells were replated on glass coverslips (13 mm diameter) for 48 h. Cells were then incubated with 10 μg/ml of *LiRecDT* for 8 h at 37 °C under a humidified atmosphere plus 5% CO_2. In the control group, the medium contained adequate amount of PBS. Cells were washed five times with PBS and fixed with 2% paraformaldehyde in PBS for 30 min at 4 °C. Cells on coverslips were incubated with 0.1 M glycine for 3 min, washed with PBS and then blocked with PBS containing 1% BSA for 30 min at room temperature (25 °C). The cells were then incubated with primary antibodies (2 μg/ml in PBS–1% BSA) for 1 h at room temperature. For antigen competition assay, the immunofluorescence protocol was the same as described above. After washing three times with PBS, the cells were subsequently incubated with secondary antibodies conjugated with fluorescein isothiocyanate. After washing, samples were mounted with Fluormont-G (Sigma) and observed under a fluorescence confocal microscope (Confocal Radiance 2,100, BioRad, Hercules, USA) coupled to a Nikon-Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division, Melville, USA).

Cell culture conditions. The cell line used in this study was MDCK (Madin Darby canine kidney epithelial cells—ATCC no. CCL-34). Cells were maintained in liquid nitrogen with a low number of passages. After thawing, cells were grown in monolayer cultures in DMEM-F12 medium containing penicillin (10,000 IU/ml) and supplemented with 10% fetal calf serum (FCS). The cultures were kept at 37 °C in a humidified atmosphere plus 5% CO_2. Release of cells was performed by treating with a 2 mM solution of ethylenediaminetetraacetic acid (EDTA) in cation-free/PBS and 0.05% trypsin for a few minutes. After counting, the cells were then resuspended in an adequate volume of medium supplemented with FCS, allowed to adhere and grow for 24 h. Cells were then evaluated in the presence or absence of *LiRecDT* (10 μg/ml and 50 μg/ml). During the experiment, the plates were photographed at 8 and 24 h using an inverted microscope (Leica-DMIL, Wetzlar, Germany), and changes in cell morphology were evaluated.

Cell cytotoxicity assays. Cytotoxicity assays were carried out on 96-well plates (TPP, Trasadingen, Switzerland) using MDCK cells, which are excellent models for in vitro cytotoxicity evaluation (Bonham et al., 2003). Cells (5×10^3 cells/well) were plated and allowed to adhere and grow for 24 h before incubation with *LiRecDT* at concentrations of 10, 25, 50, 100 and 200 μg/ml for 24 and 48 h in hexaplicate. After toxin incubation, the measurement of toxicity was performed by estimation of viability by Neutral-Red Uptake (Merck, Darmstadt, Germany) and XTT formazan-based assays (Sigma) as described by Freshney (2000) and Petrick et al. (2000). The same experimental conditions were used with control group except that the medium contained adequate amounts of vehicle (PBS) rather than *LiRecDT*. Cell viability of control group (absence of *LiRecDT*) was normalized to 100%.

A

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1        TCATGTTGCCGTACATTGTCCTAGTATTGGGGTGTTGGAGCGTCTTGTCCCAGGCTGCTCAAACAGATGATGAAGAACGC
    1      M  L  P  Y  I  V  L  V  L  G  C  W  S  V  L  S  Q  A  A  Q  T  D  D  E  E  R

                30Rec sense
           CTCGAGGCAGGTAATCGTCGGCCTATA
81       GCAGGTAATCGTCGGCCTATATGGATCATGGGGCACATGGTAAATGCCATCGGTCAGATAGACGAGTTCGTGAACCTT
   27      A  G  N  R  R  P  I  W  I  M  G  H  M  V  N  A  I  G  Q  I  D  E  F  V  N  L

159      GGAGCAAACTCCATCGAAACAGACGTGTCTTTCGATGACAATGCCAATCCTGAGTATACTTATCACGGCATTCCATGT
   53      G  A  N  S  I  E  T  D  V  S  F  D  D  N  A  N  P  E  Y  T  Y  H  G  I  P  C

237      GATTGTGGAAGGAATTGCAAGAAATATGAGAATTTTAACGATTTTCTGAAAGGTCTCCGAAGCGCCACAACACCTGGT
   79      D  C  G  R  N  C  K  K  Y  E  N  F  N  D  F  L  K  G  L  R  S  A  T  T  P  G

315      AATTCAAAGTATCAGGAAAAACTGGTCTTAGTCGTGTTCGACTTAAAGACAGGTAGCCTCTACGATAATCAAGCCAAC
  105      N  S  K  Y  Q  E  K  L  V  L  V  V  F  D  L  K  T  G  S  L  Y  D  N  Q  A  N

393      GACGCCGGAAAGAAATTGGCGAAGAATCTCTTACAACATTACTGGAACAATGGCAATAATGGTGGAAGAGCATACATA
  131      D  A  G  K  K  L  A  K  N  L  L  Q  H  Y  W  N  N  G  N  N  G  G  R  A  Y  I

471      GTGTTATCGATCCCAGACCTTAATCATTATCCACTGATTAAAGGATTCAAAGACCAGCTTACAAAGGACGGACACCCA
  157      V  L  S  I  P  D  L  N  H  Y  P  L  I  K  G  F  K  D  Q  L  T  K  D  G  H  P

549      GAGTTGATGGACAAAGTTGGACACGACTTCTCCGGAAACGACGACATCGGCGACGTTGGAAAAGCTTACAAGAAAGCA
  183      E  L  M  D  K  V  G  H  D  F  S  G  N  D  D  I  G  D  V  G  K  A  Y  K  K  A

627      GGAATAACTGGCCATATTTGGCAGAGCGATGGTATCACCAACTGTTTACCACGTGGCCTTAGTCGTGTGAACGCAGCT
  209      G  I  T  G  H  I  W  Q  S  D  G  I  T  N  C  L  P  R  G  L  S  R  V  N  A  A

705      GTGGCAAACAGAGATTCCGCAAACGGATTCATTAACAAAGTGTACTACTGGACAGTGGACAAGCGCTCAACGACCAGA
  235      V  A  N  R  D  S  A  N  G  F  I  N  K  V  Y  Y  W  T  V  D  K  R  S  T  T  R

783      GATGCACTTGATGCTGGAGTTGACGGCATAATGACCAACTACCCGGATGTTATCACTGATGTTCTCAACGAAGCCGCA
  261      D  A  L  D  A  G  V  D  G  I  M  T  N  Y  P  D  V  I  T  D  V  L  N  E  A  A

                                                  30Rec antisense
                                                ACCCACTGTAAGTTCTTTATTCCTAGGGC
861      TACAAGAAGAAATTCCGAGTTGCCACATACGACGAAAATCCATGGGTGACATTCAAGAAATAAATTCTGCAGGTTGAT
  287      Y  K  K  K  F  R  V  A  T  Y  D  E  N  P  W  V  T  F  K  K  *

939      TGTGGAAAAACACATGGCAATCTGGATTTCACGATTTTCATTGAACTTTGTTGAAAAACCAATTTGATGCGAAAAACT
1017     AAAAACTATGCACTATGGAAGCTTTGTTCAAAATATTGTTTGTTATTATTGTAAACATGTTTGAAAAAGTATTTTTCA
1095     GAAATAAATTTTGATCCATGTAAAAAAAAAAAAAAAAAAAAAAAA
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B

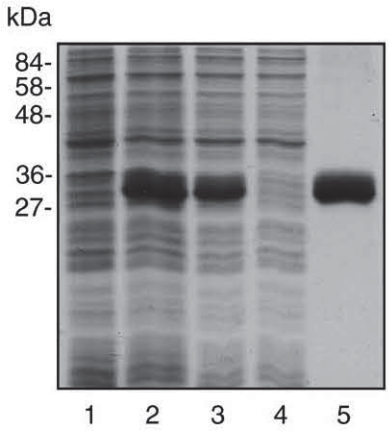

Fig. 1. Molecular cloning and expression of a functional recombinant dermonecrotic protein. (A) Nucleotide sequence of cloned cDNA for *L. intermedia* dermonecrotic toxin and its deduced amino acid sequence. In the protein sequence, the predicted signal peptide is underlined. Arrows show the annealing positions for primers 30 Rec sense and 30 Rec antisense. The asterisk corresponds to the stop codon. (B) SDS-PAGE analysis of recombinant dermonecrotic toxin expression stained by Coomassie blue dye. Lanes 1 and 2 show respectively *E. coli* BL21(DE3)pLysS cells collected by centrifugation (and resuspended in SDS-PAGE gel loading buffer) before and after 3.5 h induction with 0.05 mM IPTG. Lanes 3 and 4 depict supernatant of cells lysates obtained by freeze thawing in extraction buffer before and after incubation with Ni-NTA agarose beads, respectively. Lane 5 shows eluted recombinant protein. Molecular mass markers are shown on the left.

Statistical analysis. Statistical analysis of biochemical parameters and viability data were performed using analysis of variance (ANOVA) and the Tukey test for average comparisons GraphPad InStat program version 3.00 for Windows 2000. Mean ± standard error of mean (SEM) values were used. Significance was determined as $P \leq 0.05$.

Results

Molecular cloning and expression of a recombinant dermonecrotic toxin from the L. intermedia venom gland

Initially, we cloned and expressed a recombinant isoform of the dermonecrotic toxin (sphingomyelinase-D family), from a cDNA library of *L. intermedia* gland venom. The recombinant protein was expressed as an N-terminal 6× His-tag fusion protein in *E. coli* BL21(DE3)pLysS cells and purified from soluble fraction of cell lysates by Ni^{2+}-chelating chromatography, resulting in 24 mg/l of culture. The deduced protein from cDNA sequences resembles those obtained from several dermonecrotic toxins (sphingomyelinase-D family) of different *Loxosceles* spider species (Barbaro et al., 1996; Binford et al., 2005; Cisar et al., 1989; Pedrosa et al., 2002). BLAST search at NCBI exhibited 99% amino acid identity to *L. intermedia* dermonecrotic toxins (Kalapothakis et al., 2002, Tambourgi et al., 2004). Fig. 1A shows the cloned cDNA sequence and its deduced amino acid sequence. The longest cDNA sequence obtained is a 1139 pb molecule coding for a mature protein of calculated 31,239 Da and an isoelectric point at the region of 7.21. The cDNA revealed a 26 amino acid signal peptide before mature protein, as predicted by Nilsen's algorithm (Nilsen et al., 1997). As shown in Fig. 1B, purified recombinant toxin has SDS-PAGE mobility under reduced conditions at the region of 34 kDa.

Histopathological changes in rabbit skin induced by LiRecDT

To evaluate the functionality of dermonecrotic recombinant toxin, aliquots of crude venom and *LiRecDT* (10 μg) were injected intradermally into shaved areas of rabbit skin. The dermonecrotic lesions were checked 24 h

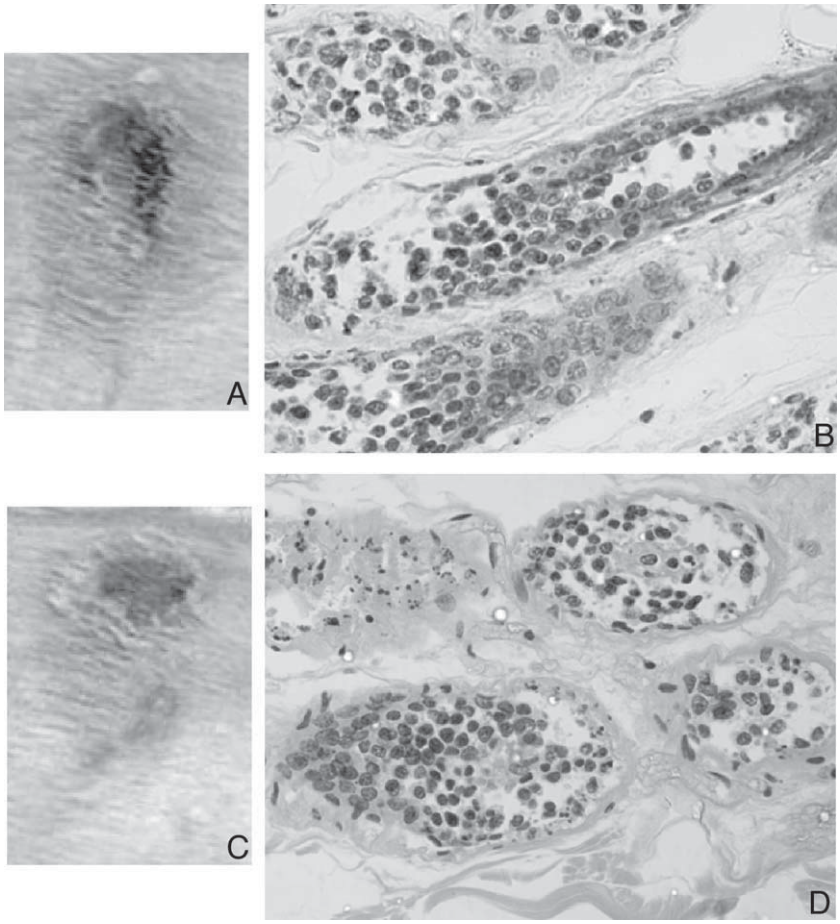

Fig. 2. Macroscopic and microscopic changes of rabbit skin exposed to crude venom and *LiRecDT*. Macroscopic visualization of dermonecrosis into the skin of a rabbit intradermally injected with 10 μg crude venom (A) and 10 μg purified *LiRecDT* (C). Light microscopic analysis of skin sections stained with HE exposed to crude venom (B) and *LiRecDT* (D) (magnification 400×). Massive inflammatory cell accumulation within blood vessels in the dermis is shown for both treatments.

after injections. Figs. 2A and B depict respectively macroscopic lesion and light microscopic analysis of biopsy from skin that received crude venom (collection of inflammatory cells in the blood vessels which represent a hallmark of dermonecrotic loxoscelism). Figs. 2C and D show the skin, which received *LiRecDT*. The results pointed to functionality of recombinant dermonecrotic toxin.

Histopathological findings in kidneys from mice that received L. intermedia recombinant dermonecrotic toxin

To ascertain the renal damage evoked by the dermonecrotic toxin of *Loxosceles* spider venom, mice were exposed i.p. to *LiRecDT* for 6 h. All animals exposed to *LiRecDT* showed alterations including lethargy, shivering and stretch attend postures suggesting physical discomfort. Light microscopic analyses of renal biopsy specimens (as depicted in Fig. 3) revealed remarkable alterations including diffuse glomerular edema, focal collapse of glomerular basement membrane, diffuse erythrocytes in the Bowman's space, diffuse hyalinization with proteinaceous material within the proximal and distal tubule lumen and diffuse vacuolar degeneration of proximal and distal tubular epithelial cells. Interestingly, neither leukocyte accumulation nor marginalization or infiltration was detected in the kidney vessels and structures.

Laboratory investigations after administration of LiRecDT

With the intent to confirm histopathological findings following *LiRecDT* treatment, we additionally determined

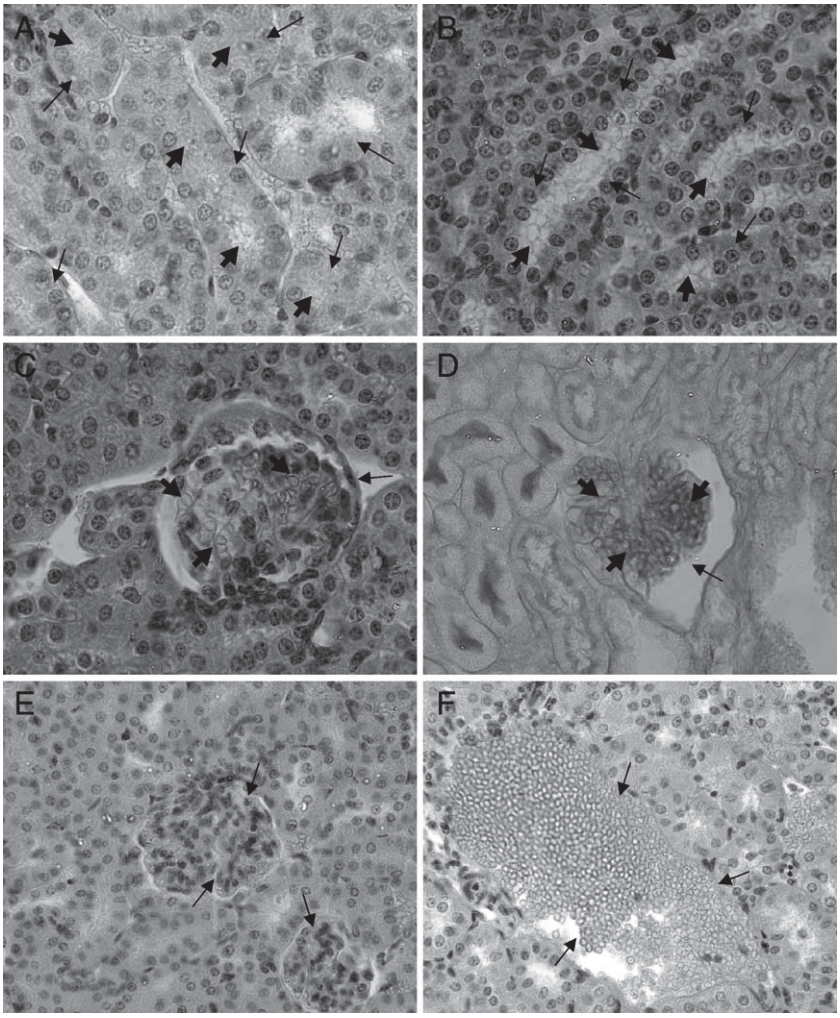

Fig. 3. Light microscopic analysis of kidneys from *LiRecDT*-treated mice. Sections of kidneys from mice treated with *LiRecDT* were stained with HE or PAS and analyzed by light microscopy. (A) Details of tubule structure (arrows) showing accumulation of proteinaceous material within the proximal and distal tubules lumen (arrowheads) (magnification 600×) (HE). (B) Details of tubule structure (arrows) showing vacuoles in the epithelial cells and vacuolar degeneration of proximal and distal tubules (arrowheads) (magnification 600×) (HE). (C) Cross-section of glomerulus (arrow) with intra-glomerular erythrocytes (arrowheads) in the Bowman's space (magnification 600×) (HE). (D) Glomerular cross-section (arrow) showing collapse of basement membranes (arrowheads) (magnification 400×) (PAS). (E) Glomerular cross-section showing edema (arrows) (magnification 400×) (HE). (F) Renal blood vessel (arrows). Note the absence of leukocytes accumulated in and around the vessel (magnification 400×) (HE).

such biochemical parameters as urinalysis and serum urea comparing LiRecDT-treated mice with control group. Serum urea was significantly increased in *LiRecDT*-treated mice (51.83 mg/dl ± 8.72 mg/dl) compared to control group (19.24 mg/dl ± 1.33 mg/dl). Similarly, hematuria and urine alkalinization were evidenced in treated animals compared to control. These findings together with histopathological analyses supported the idea of nephrotoxicity following *LiRecDT* exposure.

Evidence that LiRecDT binds to intrinsic renal components

With the aim of demonstrating that the *LiRecDT* interacts and binds directly to kidney structures, we investigated renal biopsies from *LiRecDT*-treated and control mice by immunofluorescence using an antibody that reacts with the dermonecrotic toxin. As shown in Fig. 4A, the antibody reaction produced a positive signal in renal biopsies from *LiRecDT*-treated mice but did not react with

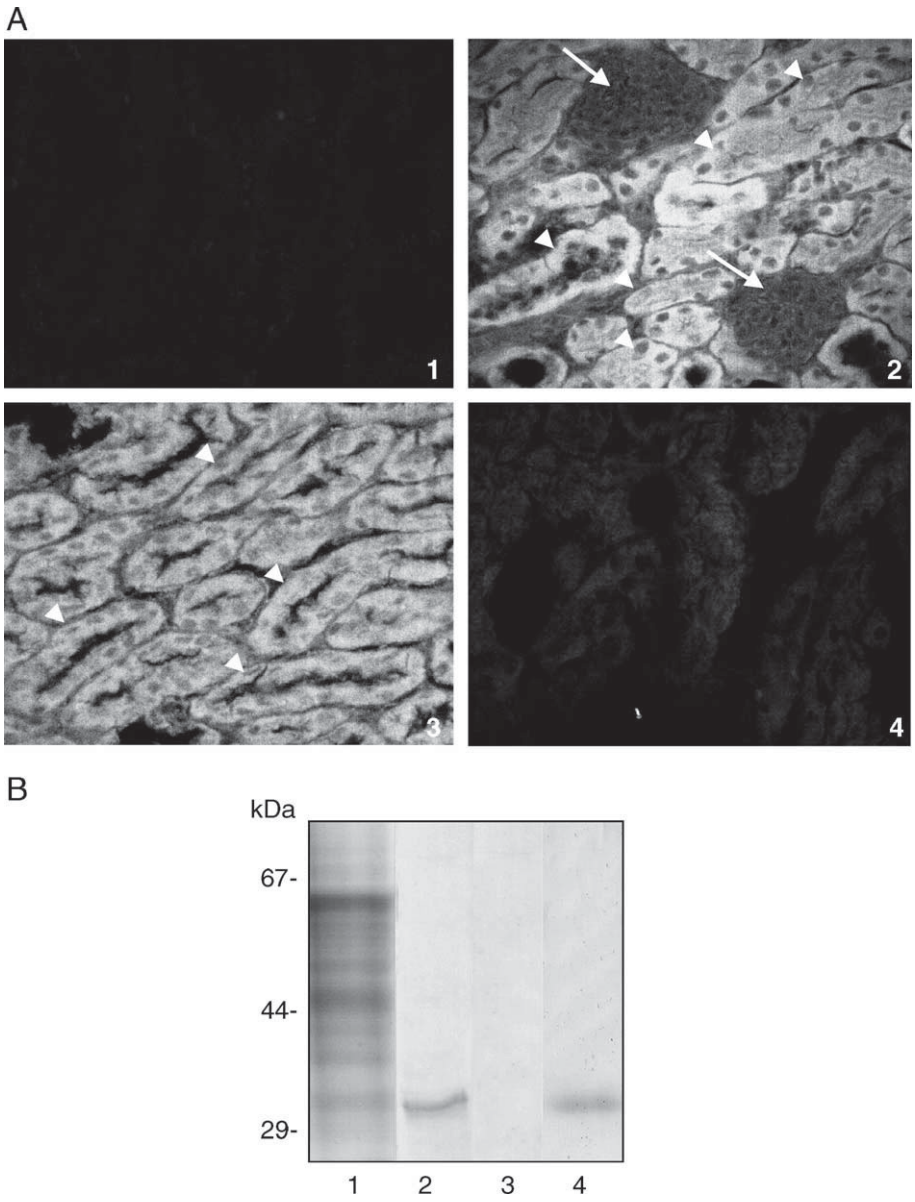

Fig. 4. Direct binding of *LiRecDT* to kidney structures. (A) Confocal immunofluorescence microscopy analysis of kidney sections from *LiRecDT*-treated mice. Cross-sectioned kidneys immunostained with antibodies against *LiRecDT*. (1) Section of a kidney from control group, which did not receive *LiRecDT*. (2 and 3) Kidney sections from *LiRecDT*-treated animals showing regions rich in glomeruli (arrows) with lower positivity and tubules (arrowheads) higher positivity for binding of *LiRecDT*. (4) Antigen competition assay in which antibodies to *LiRecDT* were previously incubated with *LiRecDT* in solution, and then a kidney section from *LiRecDT*-treated mouse was exposed to the reaction mixture under identical conditions to those described above. A great decrease in immunolabeling is shown, confirmed *LiRecDT* as "planted antigen" in kidney structures from toxin-treated mice (magnification 400×). (B) Renal extract from *LiRecDT*-treated mice (lanes 1 and 2), renal extract from control mice (absence of *LiRecDT* treatment) (lane 3) or purified *LiRecDT* (lane 4) was separated by 10% SDS-PAGE under non-reducing conditions. The gel was stained by Coomassie blue dye (lane 1) or transferred to a nitrocellulose membrane that was immunoreacted with antibodies against *LiRecDT* (lanes 2–4). Molecular protein standard masses are shown on the left of the figure.

the samples from normal mice. To confirm antibody specificity, we repeated the same immunofluorescence approach, this time incubating the antibody with *LiRecDT* (10 μg) in a solution and then exposing renal biopsies from *LiRecDT*-treated mice to this mixture (antigen competition assay). Results supported the direct binding of *LiRecDT* on the renal structures of glomerulus and tubules. Additionally, to strengthen this evidence, renal lysates from *LiRecDT*-treated mice were electrophoresed and immunoblotted with hyperimmune purified IgG which reacts to dermonecrotic toxin. As shown in Fig. 4B, we were able to detect a positive signal at the region of 33–35 kDa in the *LiRecDT*-treated lysate compared to an absence in the control lysate, confirming immunofluorescence results and identifying the dermonecrotic toxin as a direct and "planted antigen" bound to kidney structures.

Effect of LiRecDT on the morphology and viability of epithelial kidney cells

To ascertain the direct cytotoxicity of *LiRecDT* on renal cells and to support the histopathological findings, which indicated renal injuries after *LiRecDT* treatment, experiments

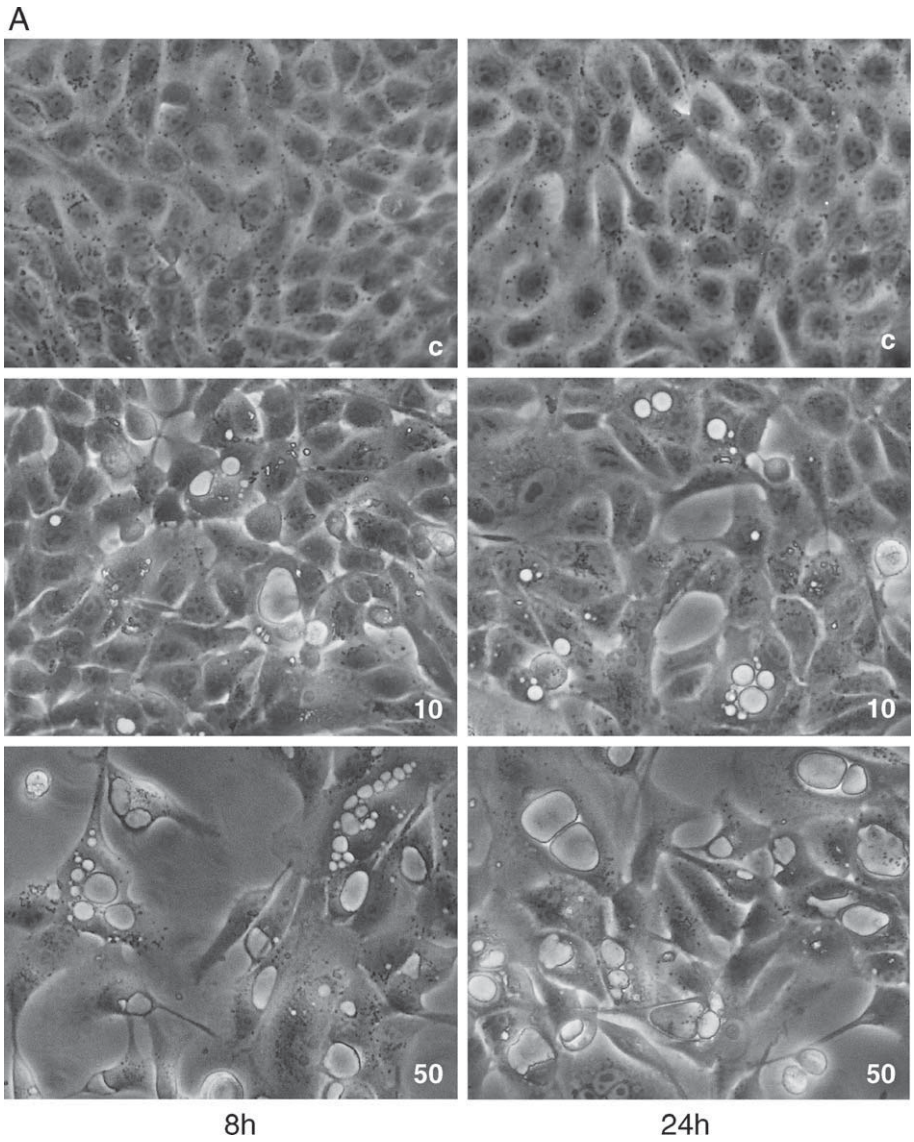

Fig. 5. Cytotoxicity assays. Effect of *LiRecDT* on the morphology of tubular epithelial cells. (A) MDCK cells exposed to *LiRecDT* were observed in an inverted microscope. The cytoplasm of the cells becomes vacuolated in a toxin concentration and time exposure manner. Identically, cell spreading appears to be impaired, and detachment from the substrate was observed. Analyses were performed at 8 and 24 h after *LiRecDT* exposure. Concentrations of purified toxin in culture medium were 10 μg/ml and 50 μg/ml (10 and 50) respectively. Control cells were analyzed in the absence of toxin (c). Cytotoxic effect of *LiRecDT* on MDCK epithelial cells analyzed by dye uptake (Neutral-Red uptake) (B) and formazan produced (XTT-based assay) (C). *LiRecDT* cytotoxic effect was determined after 24 and 48 h at indicated concentrations of purified toxin. Experiments were performed in hexaplicates, and values given are the mean ± SEM. Significance is defined as $*P < 0.05$ and $**P < 0.01$.

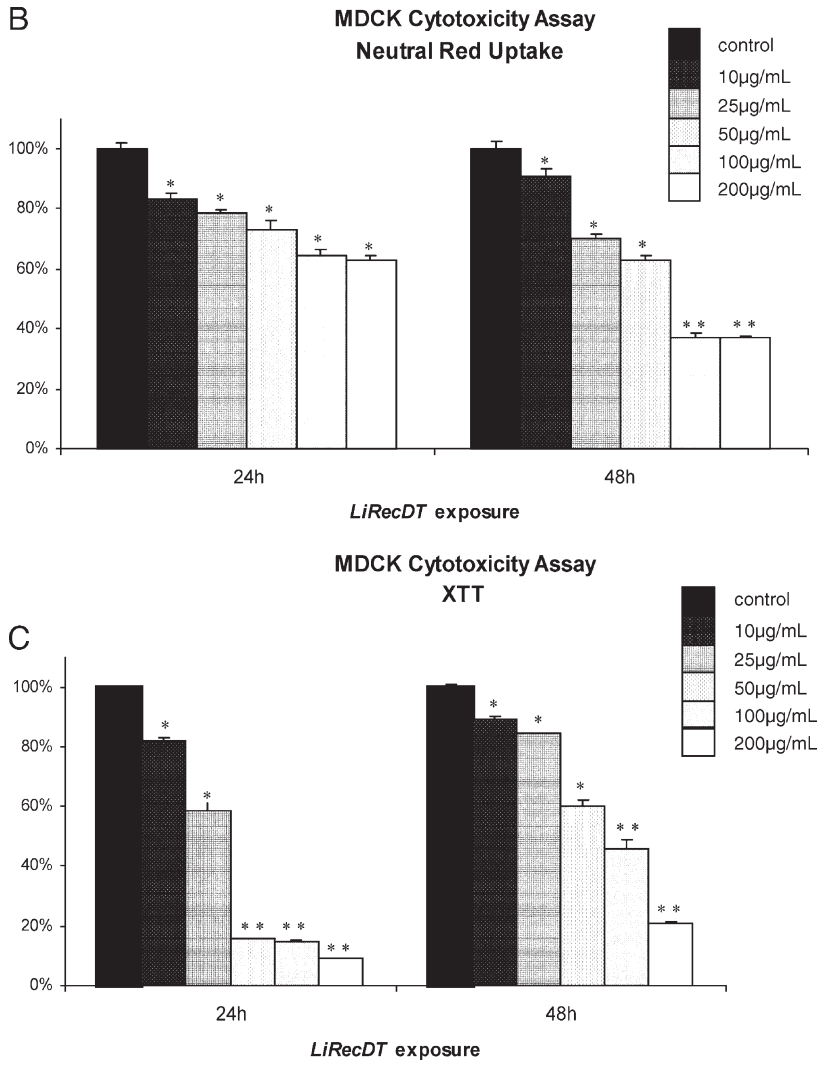

Fig. 5 (*continued*).

on morphology and cellular viability were performed using MDCK epithelial cells. As depicted in Fig. 5A, *LiRecDT* treatment induced appearance of blebs and cytoplasmic vacuolization, caused defective cell spreading and detached cells from each other and culture substratum which enhanced in a time- and toxin-dependent manner. As shown in Fig. 5B, experiments on the cellular viability (XTT and Neutral-Red Uptake) indicated a significant alteration of MDCK cell viability when compared to control cells. These in vitro experiments strengthen the idea of dermonecrotic toxin nephrotoxicity.

The LiRecDT binds to MDCK epithelial cells

With the objective of corroborating the direct cytotoxicity of dermonecrotic toxin upon renal cells, we looked for the direct binding of *LiRecDT* on MDCK cells in culture. For this purpose, toxin-treated and control cells were submitted to an immunofluorescence experiment using an antibody, which reacts with dermonecrotic toxin. As shown in Fig. 6, *LiRecDT* binds to MDCK cells, producing an immunofluorescent pattern of deposition on the cell surface.

Discussion

Loxoscelism, the term representing accidents and envenomation involving spiders of *Loxosceles* genus (brown spider), has been reported world-wide (Futrell, 1992; da Silva et al., 2004). The clinical features of brown spider bites are an image of necrotic skin lesions which can also be accompanied by a systemic involvement including weakness, vomiting, fever, convulsions, disseminated intravascular coagulation, intravascular hemolysis and renal failure. Severe systemic loxoscelism is much less common than the cutaneous form, but it may be the cause of clinical complications and even death after envenomation (Futrell, 1992; da Silva et al., 2004). Reactions to brown spider bites are influenced by the victim's health, degree of obesity and

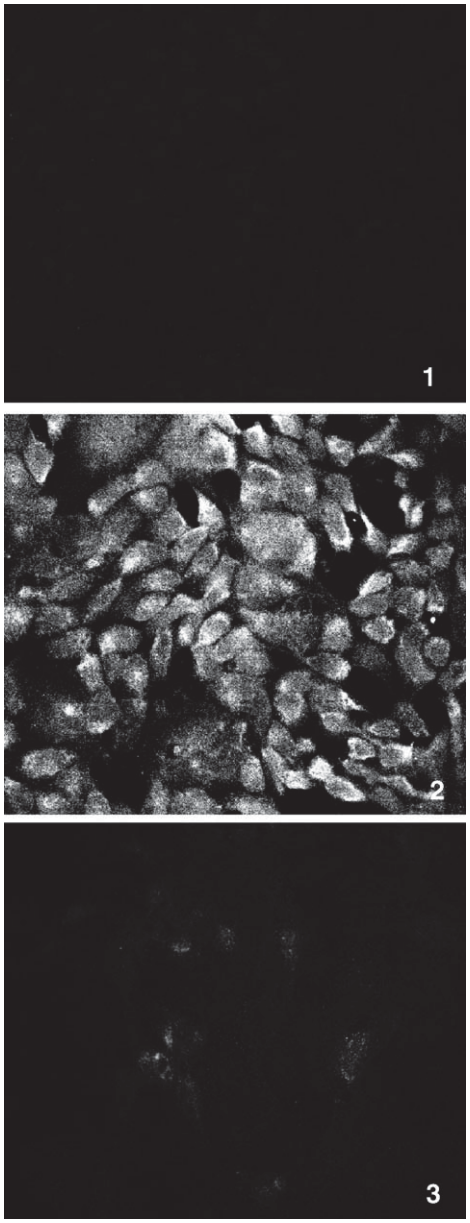

Fig. 6. *LiRecDT* binding to MDCK epithelial cells. Confocal immunofluorescence microscopy analysis from *LiRecDT*-treated MDCK cells. (1) Cells, which did not receive *LiRecDT* treatment, incubated with antibodies for toxin and specific secondary fluorescent conjugate. (2) *LiRecDT* deposition and binding on cell surface of MDCK cells was detected by confocal immunofluorescence labeling by using specific antibodies for toxin. (3) Result of an antigen competition assay in which antibodies to dermonecrotic toxin were previously incubated with purified *LiRecDT* in solution, and the mixture was exposed to toxin-treated MDCK cells under conditions identical to the experiment of lane 2. The absence immunostaining confirmed the specificity of the reaction and *LiRecDT* as "planted antigen" on MDCK cell surface.

localization of the bite among other factors (Sams and King, 1999; da Silva et al., 2004; Vetter and Bush, 2002).

The nephrotoxic effects of *Loxosceles* spider venom are demonstrated based on the clinical and laboratory features observed in some victims, which can include elevated creatine kinase levels, hematuria, hemoglobinuria, proteinuria and shock (Bey et al., 1997; França et al., 2002; Lung and Mallory, 2000; Williams et al., 1995). Additionally, Luciano et al. (2004) were able to show deposition and binding of brown spider venom toxins along the tubular and glomerular structures and a consequent cytotoxicity in renal tissue of mice. Hematological disturbances such as hemolytic anemia and disseminated intravascular coagulation, as well as nephrotoxicity secondary to complications of dermonecrotic lesions have been postulated as pathological processes that may lead to renal failure (Futrell, 1992; Lung and Mallory, 2000; Williams et al., 1995). However, there is no direct experimental evidence confirming such a hypothesis. In addition, histopathological findings after envenomation of mice (an animal model which does not develop dermonecrotic lesions caused by *Loxosceles* spider venom) (Futrell, 1992; da Silva et al., 2004), together with the binding of venom toxins to renal structures and the apparent absence of hemoglobin in the proteinaceous materials inside the Bowman's space and tubules detected after venom exposure (Luciano et al., 2004), strongly support a direct nephrotoxicity activity of venom toxins and the hypothesis of "planted toxins" to intrinsic components of renal structures. This idea corroborates with several reports that evidence "planted antigens" including viral, parasitic products, bacteria and drugs such as etiological agents to renal injuries (Cotran et al., 1999).

The presence of a 30 kDa protein in renal lysates from crude-venom-treated mice was indicative of dermonecrotic toxin involvement in renal injuries caused by *Loxosceles* venom (Luciano et al., 2004). This assumption was also supported by the cytotoxic activity of dermonecrotic toxin upon erythrocytes and platelets (Futrell, 1992; da Silva et al., 2004) and by its lethal activity (Barbaro et al., 1996).

In this study, we cloned, expressed and purified a recombinant isoform of the dermonecrotic toxin from *L. intermedia* gland venom. This recombinant toxin showed functionality as observed by dermonecrosis and an inflammatory response upon rabbit skin, which was similar to those activities evoked by crude venom. *LiRecDT* is one of several dermonecrotic toxin isoforms present in the venom (Tambourgi et al., 2004). It seems that each isoform of dermonecrotic toxin causes noxious activities, and the effect induced by crude venom represents a family synergism. Additionally, venom toxins other than the dermonecrotic family can contribute to dermonecrosis size and intensity. Metalloproteases described in the venom (Feitosa et al., 1998), hyaluronidases (Futrell, 1992; Young and Pincus, 2001; da Silva et al., 2004) and even complications from secondary infections (Monteiro et al., 2002) could be involved in dermonecrosis.

The glomerular and tubular damage observed in the *LiRecDT*-intraperitoneal-treated mice were similar to those induced by crude venom (Luciano et al., 2004). The azotemia detected by the increase in serum urea and such biochemical urine changes as alkalinization and hematuria together with histopathological studies followed *LiRecDT*

exposure evidenced the nephrotoxicity caused by the dermonecrotic toxin.

In contrast to the cutaneous lesion in which polymorphonuclear leukocytes play an essential role in the pathogenesis (an aseptic coagulative tissue necrosis) (Elston et al., 2000; Futrell, 1992; Ospedal et al., 2002; da Silva et al., 2004), the renal injury evoked by dermonecrotic toxin is not associated with inflammatory changes as in immune complex nephritis which is caused by deposition of exogenous antigens following some bacterial and viral infections (Cotran et al., 1999; Tisher and Brenner, 1994). In situ immune complex deposition can also be discarded because biopsies were collected 6 h after *LiRecDT* exposure, mouse pre-immune serum did not react with crude venom or recombinant dermonecrotic toxin (which could suggest natural immunoglobulins to this toxin) and immunofluorescence using anti-mouse IgG was negative in biopsies from toxin-treated mice (data not shown).

Additionally, immunoblotting analysis of renal lysates from *LiRecDT*-treated mice using dermonecrotic toxin antibodies identified the presence of this toxin at the 33–35 kDa region as direct ligand of renal intrinsic structures. This, together with the immunofluorescence results of toxin-treated renal biopsies, confirmed this protein as an "exogenous planted antigen" along the kidney structures.

The glomerular barrier function is dependent on the molecular mass of proteins (molecules with mass lower than 70 kDa are more permeable than larger proteins), as well as molecular charge of proteins (anionic molecules tend to be less permeable and are repulsed by anionic moieties present within the renal structures) (Cotran et al., 1999; Farquhar, 1991). We postulated based on the physicochemical properties of *LiRecDT* (33–35 kDa and pI 7.2), together with its water solubility, that these properties account for the binding of this molecule to kidney structures and consequent nephrotoxicity.

Dermonecrotic toxin nephrotoxicity was additionally proved by confocal immunofluorescence with antibodies that react to this molecule and by using MDCK epithelial cells in culture, which demonstrated the direct toxin binding on the cell surface. In addition, experiments using MDCK cells treated in culture with *LiRecDT* showed a potent toxin activity, particularly in the disturbance of cell morphology, which induced the appearance of vacuoles in cytoplasm, changed their spreading aspect and caused defective cell–cell and culture substratum adhesion (as shown by Collares-Buzato et al. (2002) using snake venom toxins).

Likewise, the toxin also inhibited cellular viability in a concentration- and time-dependent manner, further demonstrating a toxin direct cytotoxicity. The venom concentration to which victims would be exposed following envenomation depends on such factors as size and sex of spiders (females inject more venom than males). The total venom volume injected is about 4 μl and contains 65–100 μg of proteins (Sams et al., 2001; da Silva et al., 2004). In fact, even 10 μg and 25 μg of toxin have shown cytotoxicity upon MDCK cells and dermonecrosis into the rabbit skin. These low experimental *LiRecDT* concentrations resemble the toxin levels that would be seen after a typical *Loxosceles* envenomation. The molecular mechanism by which dermonecrotic brown spider toxin causes renal injuries is currently unknown. Since mice were used (an animal model which does not develop dermonecrotic lesions) (Futrell, 1992; da Silva et al., 2004), we can rule out nephrotoxicity in vivo secondary to complications of dermonecrosis. This conclusion is similarly corroborated by the toxin direct cytotoxicity upon MDCK cells in vitro. Likewise, some reports have indicated the participation of a serum amyloid P plasma component of adult animals but not from fetal plasma on the dermonecrotic toxin-dependent platelet activation and aggregation (Gates and Rees, 1990). Since our data indicated dermonecrotic toxin cytotoxicity on MDCK cells in the presence of fetal calf serum (culture medium), this last hypothesis can be discarded.

Experiments using endothelial cells treated in culture with brown spider venom show a potent endothelial cell agonist activity of the venom (dermonecrotic toxin), which induces endothelial cell release of macrophage colony-stimulating factor and interleukine-8, causing an exacerbated inflammatory response (Patel et al., 1994). Tambourgi et al. (1998) postulated that renal damage induced by *Loxosceles* spider venom putatively could be mediated by cytokine mediators. On the basis of direct dermonecrotic toxin cytotoxicity upon MDCK cells in vitro, the absence of inflammatory leukocytes in the renal histological analysis, but with a marked damage to kidney structures, we can consider this hypothesis with restriction.

On the basis of the above results, we have identified a toxin responsible for cellular and pathological alterations which causes nephrotoxicity after accidents involving *Loxosceles* spiders.

Acknowledgments

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Os acidentes com aranhas do gênero *Loxosceles* estão classicamente associados ao desenvolvimento de lesões dermonecróticas e manifestações sistêmicas, dentre as quais se destacam os distúrbios hematológicos e a falência renal aguda. O efeito nefrotóxico severo observado nas vítimas é menos comum que a dermonecrose no local da picada, no entanto é a complicação sistêmica a qual se atribui a maioria dos óbitos decorrentes de envenenamento por aranha marrom (FUTRELL, 1992; DA SILVA *et al.*, 2004). Martinez-Vargas (1987) relata que 62% dos casos de loxoscelismo cutâneo-visceral, o rim encontra-se comprometido, atingindo o estado de insuficiência renal aguda (IRA). Pacientes que não realizaram diálise perfazem a taxa mais elevada de mortalidade, apresentando padrões histológicos de necrose tubular aguda. Achados laboratoriais dos pacientes descrevem hemoglobinúria, hematúria e proteinúria (FUTRELL, 1992; LUNG E MALLORY, 2000), provavelmente reflexos da hemólise intravascular e coagulação intravascular disseminada induzidas pelo envenenamento (FUTRELL, 1992; WILLIAMS *et al*., 1995; LUNG E MALLORY, 2000). Contudo, não existem evidências experimentais para tais hipóteses, além do fato que a nefrotoxicidade pode ser em virtude da ação direta dos componentes do veneno sobre as células renais e membranas basais tubulares e glomerulares, como já foi descrito para venenos de outros animais (OHSAKA *et al.*, 1973; WILLINGER *et al.*, 1995; DOS REIS *et al*., 1998; RAHMY *et al.*, 2000; DE CASTRO *et al*., 2004).

O primeiro trabalho publicado (LUCIANO *et al*., 2004) possibilitou a observação da intensidade do efeito tóxico do veneno de aranha marrom sobre as estruturas renais por meio da avaliação morfológica de biópsia renal de camundongos envenenados experimentalmente, além de dados laboratoriais dos mesmos. A atividade nefrotóxica do veneno loxoscélico é revelada pelas alterações do epitélio tubular e dos glomérulos, utilizando microscopia de luz (figura 1) e eletrônica de transmissão (figura 2). De modo geral, tanto a porção medular quanto cortical do tecido renal apresenta um padrão complexo de nefrotoxicidade, onde se destacam a vacuolização das células epiteliais, o colapso das membranas basais glomerulares e a deposição de material proteináceo no epitélio tubular e na cápsula de Bowman. Ultraestruturalmente são observados sinais de destruição celular por necrose, tais como bolhas na membrana citoplasmática, injúria mitocondrial e presença de autofagossomos. Vale ressaltar a ausência de infiltrado leucocitário no tecido renal, ao contrário

do que é observado na lesão cutânea dermonecrótica, na qual os leucócitos polimorfonucleares estão presentes contribuindo para um quadro inflamatório importante (SMITH E MICKS, 1970; FUTRELL, 1992; PATEL *et al*., 1994; OSPEDAL *et al*., 2002; DA SILVA *et al*., 2004). Durante o dano renal, não houve infiltrado leucocitário, logo sugerindo que não se trata de doença inflamatória.

Outro fato interessante é que a hemólise intravascular normalmente relacionada ao efeito nefrotóxico não parece estar presente no modelo estudado, sabe-se que o veneno de aranha marrom induz hemólise intravascular (DILLAHA *et* al., 1964; MURRAY E SEGER, 1994; WILLIAMS *et al.*,1995) e possui ação hemolítica direta sobre eritrócitos (FORRESTER *et al*., 1978; MORGAN *et al.*, 1978), no entanto não se observou deposição de hemoglobina nas estruturas renais (figura 3), como já observado em ratos (COUTINHO, 1996) e os dados laboratoriais não apontaram sua excreção (tabela 2) como poderia se esperar. Esses resultados estão em concordância com Futrell (1992) que relaciona distúrbios hematológicos como fatores secundários à injúria renal e aponta algumas espécies animais como sendo mais susceptíveis à hemólise (humanos e suínos) do que outras (coelhos e cobaias). Não se pode afirmar que não há envolvimento da ação hemolítica com as disordens renais, visto que o veneno não induz dermonecrose e hemólise em camundongos, no entanto foi extremamente danoso às estruturas renais o que reforça a idéia de uma ação direta das toxinas loxoscélicas ao tecido renal.

O emprego de anticorpos como bioferramentas no estudo de venenos é amplamente difundido em Toxinologia, desde a utilização como antisoros até a detecção para fins diagnósticos (FALKENBERG *et al.*, 1984; CLOT-FAYBESSE *et al.*, 1999; GATHUMBI *et al*., 2001; GUILHERME *et al.*, 2001; CASADEVALL, 2002; ALVARENGA *et al.*, 2003; BENBROOK, 2002; RAWEERITH E RATANABANANGKOON, 2005). A produção e utilização de anticorpos, que reconhecem os componentes do veneno de aranha marrom, confirmaram a hipótese levantada quanto à interação direta do veneno ao tecido renal. Os anticorpos produzidos possibilitaram a visualização da ligação das toxinas loxoscélicas às estruturas renais por meio de reações de imunofluorescência (figuras 4 e 5) e de *western blotting* do extrato renal de camundongos expostos ao veneno de aranha marrom (figura 6). Ensaios de competição (figura 4D e

6B-5) excluíram a possibilidade de inespecificidade da reação nos ensaios realizados. Os resultados condizem com dados da literatura que descrevem a ligação de moléculas exógenas conhecidas como “antígenos plantados”, tais como produtos bacterianos, antígenos virais, drogas e outros venenos, em constituintes do tecido renal como agentes responsáveis pela injúria renal (ABUELO, 1990; COUSER, 1999; KERJASCHKI, 1996; COTRAN *et al*., 1999). Não foram encontradas evidências em nosso trabalho do envolvimento de componente imune relacionado ao mecanismo de nefrotoxicidade (TAMBOURGI *et al*., 1995), pois não foram observadas alterações dos níveis de C3 (tabela 3) e não foram detectados depósitos de imunoglobulinas quando realizados ensaios utilizando anticorpos conjugado anti-IgG de camundongo (dados não mostrados). Portanto, sugerindo que o veneno liga-se ao rim e constitui um agente primário da lesão renal evidenciada.

A microscopia confocal demonstrou a colocalização da imunomarcação das toxinas do veneno com constituintes da membrana basal (colágeno tipo IV e laminina) o que corrobora com a idéia do efeito citotóxico direto do veneno (figura 5). A membrana basal nos rins participa da filtração glomerular e da organização e adesão dos demais tipos celulares necessários ao processo de formação da urina (podócitos, epiteliais e endoteliais) (FAQUHAR, 1991). O veneno é composto por uma mistura de enzimas hidrolíticas que degradam tais constituintes e, por conseguinte, explicando a citotoxicidade observada nas células pela alteração da membrana basal e desorganização do tecido.

A eletroforese bidimensional mostrou que o veneno é rico em toxinas catiônicas e de baixa massa molecular (figura 7A). Em “imunoblotting” utilizando anticorpos para toxinas do veneno, o extrato renal proveniente de camundongos tratados com veneno, foi possível detectar uma toxina na região de 30kDa que estava ligada ao rim (figura 7B). Esses resultados sugerem para a hipótese de que possíveis toxinas como “antígenos plantados” interagem com o tecido renal com afinidade a compostos aniônicos (proteoglicanos na membrana basal) presentes nas regiões glomerulares (VOGT *et al*., 1980; FAQUHAR, 1991; COTRAN, 1999). Além de que a barreira de filtração é permeável a maioria das moléculas do veneno, que possuem baixa massa molecular (<70kDa), permitindo assim que as toxinas primeiramente se liguem na membrana basal e depois nas estruturas tubulares. Desse modo, um possível mecanismo celular e molecular foi apresentado a fim de aprofundar os

conhecimentos sobre a citotoxicidade do veneno de aranha marrom sobre o tecido renal.

O veneno de aranha marrom tem sido descrito como ativador da agregação plaquetária, por atuar sobre os eritrócitos causando hemólise intravascular (FUTRELL, 1992; WILLIAMS *et al.*, 1995; DA SILVA *et al.*, 2004), possuir efeito inibitório sobre neutrófilos *in vitro* (MAJESTIK *et al.*, 1977), bem como, pelos efeitos citotóxicos em células endoteliais de veia de cordão umbilical humano e de vaso sangüíneo de coelho (PATEL *et al.*, 1994; VEIGA *et al.*, 2001a). Zanetti *et al*. (2002) e Ospedal *et al*. (2002) demonstraram por achados histopalógicos os efeitos nocivos do veneno de aranha marrom sobre a pele e vasos sangüíneos de coelhos. O veneno ainda altera a integridade da membrana basal de EHS (Engelbreth-Holm-Swarm) (VEIGA *et al.*, 2000b), além dos danos descritos anteriormente às células e estruturas de tecido renal de camundongo (LUCIANO *et al*., 2004).

Portanto, no segundo artigo deste trabalho, baseado em tais atividades citotóxicas, nós prospectamos ações similares sobre diferentes linhagens celulares tumorais (CREPLIVE, 2004). A viabilidade e a proliferação celular foram avaliadas pelo ensaio de exclusão por Azul de Trypan e pelo método de MTT, respectivamente. Embora o tratamento de veneno tenha interferido na morfologia de todas as linhagens celulares estudadas (figura 1) que se tornaram arredondadas e em sua maioria se soltaram do substrato de cultura, não houve alteração significativa da viabilidade celular nas condições avaliadas, com exceção das células HeLa, as quais houve uma redução de 50% depois 72 horas de exposição de veneno (100μg/mL). A taxa de proliferação foi muito variável entre as linhagens avaliadas e dependente da concentração utilizada.

A literatura descreve ação semelhante de outros venenos de artrópodes sobre linhagens tumorais que demonstraram pouca atividade sobre a viabilidade celular, enquanto que sobre o efeito proliferativo, houve maior intensidade (COHEN E QUISTAD, 1998; PEREIRA-BITTENCOURT *et al*., 1999; DOMINGOS *et al.*, 2003).

Utilizando técnicas de imunofluorescência e microscopia confocal para avaliar alterações morfológicas desencadeadas pelo veneno, foi observado perturbação dos pontos de adesão focal e desorganização dos filamentos de actina (figura 2), bem como, a ligação das toxinas do veneno na superfície

celular (figura 3) da linhagem MCF-7. Além disso, o tratamento de veneno também hidrolisou a matriz extracelular como demonstrado pela coloração de PAS (figura 4). Alterações de citoesqueleto poderiam ser explicadas não somente pela ação do veneno sobre os componentes da matriz extracelular, como já previamente observado em diferentes modelos celulares (FEITOSA *et al.*, 1998; VEIGA *et al.*, 2000b, 2001a, 2001b; ZANETTI *et al*., 2002; LUCIANO *et al.*, 2004), mas também pela ação na superfície celular em receptores e/ou proteínas acessórias do citoesqueleto. Os resultados apontaram o efeito citotóxico do veneno de aranha marrom como concentração e tempo-dependentes sobre a morfologia das células tumorais, em especial sobre as estruturas adesivas das células e abrem a possibilidade da utilização das toxinas do veneno como ferramentas viáveis para a pesquisa de Biologia Celular, já que provocam alterações importantes da organização do citoesqueleto e da manutenção da matriz extracelular, ambos indispensáveis para inúmeros processos biológicos, tais como os fenômenos de migração e adesão celular (ALBERTS *et al*., 2002).

O terceiro artigo do presente trabalho permitiu o aprofundamento do conhecimento do efeito citotóxico do veneno de aranha marrom, associando a ação *in vivo* e *in vitro* de uma toxina dermonecrótica recombinante *LiRecDT* (família da esfingomielinase-D) sobre o modelo renal. Desse modo, verificamos a atividade da toxina recombinante indutora de lesão dermonecrótica como observado por outros autores (ARAUJO *et al*., 2003; TAMBOURGI *et al*., 2004) e relatamos os seus possíveis efeitos citotóxicos sobre o tecido renal. A injúria renal com veneno bruto descrita no primeiro artigo (LUCIANO *et al.*, 2004) foi novamente reproduzida com a *LiRecDT* que possui propriedades físico-químicas (33-35kDa e pI $\cong$ 7,2) (figura 1) semelhantes a região de 30kDa descrita anteriormente (LUCIANO *et* al., 2004), presente no extrato renal de camundongo exposto ao veneno. Os achados histopatológicos observados (figura 2) foram semelhantes aos obtidos com o veneno total, destacando um efeito maior sobre as células e estruturas tubulares as quais apresentaram acúmulo de material proteináceo no lúmen dos túbulos e vacuolização das células epiteliais tubulares. Os glomérulos apresentaram edema e colapso das membranas basais capilares, além da presença de eritrócitos extravasculares, entretanto, não houve acúmulo de material eosinofílico no lúmen glomerular. A ausência de infiltrado leucocitário, em contraste com a intensa presença de

polimorfonucleares na região da lesão dermonecrótica (FUTRELL, 1992; ELSTON *et al.*, 2000; OSPEDAL *et al.*, 2002; DA SILVA *et al.*, 2004), o que reforça a idéia de ação direta da toxina sobre as estruturas renais em detrimento de um possível efeito inflamatório como poderia ser sugerido. Tais resultados descrevem dano renal com destruição necrótica das células renais que aparentemente não está associada à nefrite por deposição de imunocomplexos (TISHER E BRENNER, 1994; COTRAN *et al.*, 1999), pois não houve detecção de imunoglobulinas quando realizado ensaio de imunofluorescência com conjugado anti-IgG de camundongo (dados não mostrados). No extrato renal de camundongos expostos a *LiRecDT* também foi detectada a presença da toxina recombinante por imunoreação como ligante direto da membrana de células renais, suportando a hipótese levantada em Luciano *et al.* (2004) de toxinas loxoscélicas serem “antígenos plantados” na superfície celular renal.

A utilização de linhagem celular epitelial renal (MDCK) possibilitou a observação de efeito nefrotóxico *in vitro* o que descartou a interferência do componente imunológico da análise, as células apresentaram alterações morfológicas de vacuolização citoplasmática e de espalhamento celular quando tratadas com *LiRecDT* (figura 4A) que podem estar relacionadas a desestabilização da adesão celular, como já observado por Collares-Buzatto *et al*. (2002) com toxinas de *Bothrops moojeni* em cultura celular de MDCK.

Nos ensaios de citotoxicidade utilizando os métodos de captação por endocitose de corante (vermelho neutro) e de XTT, observou-se que a viabilidade celular é reduzida de maneira diretamente proporcional a concentração e tempo exposição à *LiRecDT.* Tais observações podem ser relacionadas com alteração de permeabilidade celular frente à toxina recombinante, fato reportado em ensaios com outras toxinas (PELICIC *et al.*, 1999*;* WILLINGER *et al*., 1995; COLLARES-BUZATTO *et al.*, 2002; DONELLI *et* al., 2003; PETIT *et al*., 2003). A detecção de *LiRecDT* em cultura de MDCK, com anticorpos que a reconhecem por ensaio de imunofluorescência em microscopia confocal, corrobora para ação direta do veneno de aranha marrom sobre o tecido renal. A possível participação de substâncias do plasma de animais adultos e, conseqüente deposição da proteína amilóide P sérica, foi descartada, pois o meio de cultura utilizado contém soro fetal, logo não houve interferência desse componente como descrito por Gates e Rees (1990) para

ativação e agregação plaquetária. Tambourgi *et al.* (1998b) associa o efeito nocivo sobre a função renal como resposta secundária à ativação de endotélio por leucócitos e à liberação de mediadores inflamatórios (p.ex., interleucina-8). Contudo, a ausência de células inflamatórias nos achados histopatológicos e a atividade citotóxica sobre MDCK em cultura fornecem evidências concretas de que o efeito da toxina dermonecrótica, assim como também do veneno bruto, é devido a sua ação direta no tecido renal, logo a hipótese de ação estritamente secundária a um evento inflamatório deve ser observada com restrição.

5. CONCLUSÃO

A produção e utilização de anticorpos permitiram a detecção das toxinas do veneno bruto e da *LiRecDT* no tecido renal de camundongos e nas linhagens celulares estabelecidas MDCK (epitélio tubular renal) e MCF-7 (adenocarcinoma de mama). Os anticorpos produzidos foram biorreagentes no estudo das atividades nocivas do veneno, tanto em experimentos de imunofluorescência quanto em reações de "western blotting", apresentando alta afinidade aos componentes do veneno de aranha marrom.

A citotoxicidade induzida *in vitro* em ensaios com diversas linhagens tumorais foi concentração e tempo-dependentes. De modo geral, a viabilidade celular foi pouco alterada (com exceção da linhagem HeLa) e o efeito proliferativo observado foi bastante variável. O veneno de aranha marrom foi detectado na superfície celular e as alterações morfológicas foram bastante evidentes, provavelmente associadas a desorganização do citoesqueleto e degradação da matriz extracelular, o que demonstra o potencial do veneno de aranha marrom no estudo dos processos de adesão e migração celular em Biologia Celular.

A nefrotoxicidade desencadeada durante os acidentes com aranhas do gênero *Loxosceles* foi abordada *in vivo* e *in vitro*, foram evidenciadas alterações histopatológicas consistentes com necrose das estruturas tubulares e glomerulares em amostras de tecido de camundongos experimentalmente envenenados, como também alterações (concentração e tempo-dependentes) da morfologia (vacuolização citoplasmática e alteração do padrão de espalhamento celular) e da viabilidade celular da linhagem celular epitelial renal MDCK exposta experimentalmente a *LiRecDT.*

A ligação direta das toxinas loxoscélicas foi demonstrada em amostras de tecido de camundongos experimentalmente envenenados e de células MDCK expostas a *LiRecDT* em ensaios de imunofluorescência e "western blotting". O efeito inflamatório como causa principal da injúria renal, destacado na lesão dermonecrótica no local da picada, foi descartado nos experimentos descritos, visto que não foram observados leucócitos no tecido renal. Além disso, a utilização de cultivo celular para avaliação da citotoxicidade da toxina dermonecrótica também excluem tal interferência.

A detecção de toxinas loxoscélicas catiônicas e de massa molecular em torno de 30kDa, inicialmente com veneno bruto e, subseqüentemente, com a toxina recombinante que contém as mesmas propriedades de carga e massa

molecular, reforçou a hipótese de um mecanismo primário e direto do veneno sobre a disfunção renal desencadeada no loxoscelismo.

Os resultados apresentados nesta dissertação cumprem os objetivos propostos no projeto e abrem inúmeras possibilidades na compreensão dos eventos relacionados à citotoxicidade do veneno de aranha marrom. Os artigos científicos provenientes desta linha de pesquisa contribuíram para o aprofundamento dos prováveis mecanismos fisiopatológicos envolvidos nos acidentes com aranhas do gênero *Loxos*celes.

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